Early-life immunity and susceptibility to Mycobacteria

Erin Lesley Logan

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Supervisor: Associate Professor William GC Horsnell

Co-supervisors: Professor Mark Hatherill, Professor Adam F Cunningham

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Name: Erin Lesley Logan
Student number: LGNERI001
Signature: Signed by candidate

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Thesis abbreviations

Abbreviation	Explanation
AP	alkaline phosphatase
APC	antigen presenting cell
ATPase	adenosine triphosphatase
BCG	bacille Calmette-Guérin
BCR	B cell receptor
CD	cluster of differentiation
CFP-10	10kDa culture filtrate protein
CFU	colony-forming units
ELISA	enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization
ESAT-6	6kDa early secretory antigenic target
FACS	fluorescence-activated cell sorting
FcR	Fc receptor for antibody
FcRn	Neonatal Fc receptor
FS	filter sterilized
GFP	green fluorescent protein
HIV	Human Immunodeficiency Virus
HLA	human leukocyte antigen
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IL-4Rα	interleukin 4 receptor-α
IN	intranasally
IP	intraperitoneally
IQR	interquartile range
LAMP-1	lysosomal-associated membrane protein 1
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MST	mediastinal
Mtb	*Mycobacterium tuberculosis*
MVA85A	modified vaccinia virus Ankara expressing antigen 85A
NaCl	sodium chloride
NaOH	sodium hydroxide
NOS2	nitric oxide synthase 2
OADC	Oleic Albumin Dextrose Catalase
OD	optical density
PBMC	peripheral blood mononuclear cell
PNP	p-Nitrophenyl phosphate
PPD	purified protein derivative
SC	subcutaneously
STAT6	signal transducer and activator of transcription 6
TB	tuberculosis
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th	helper $CD4^+$ T cell
TLRs	toll-like receptors
TNF	tumour necrosis factor
Treg	regulatory $CD4^+$ T cell

Abstract

The naïve and not-yet developed infant immune system exhibits heightened susceptibility to external factors (e.g. pathogens), and is shaped by these and others, such as maternal immunity. However, we do not yet fully understand their impact on development of infant immunity. A better understanding of these effects would benefit children world-wide, but especially those in low-middle income countries (LMIC), where increased exposure to pathogens due to poorer living conditions highlights the necessity of robust early-life immunity. *Mycobacterium tuberculosis (Mtb)* and helminths are pathogens co-endemic in many LMIC and cause significant morbidity and mortality in children. Infant immune responses to these pathogens, whether during standalone infection, co-infection or resulting from maternal infection are not fully understood. To contribute to this knowledge gap, we investigated early-life immune responses, how they relate to childhood *Mtb*/helminth infection and how they are affected by maternal infectious history and immunity.

Analysis of clinical humoral responses revealed total IgG that increased significantly between baseline and tuberculosis (TB) investigation in infants who did not acquire *Mtb* infection; these infants also exhibited raised levels of measles-specific IgG and BCG-specific IgG2. No active helminth infections were detected, but the presence of *Ascaris lumbricoides*- and *Trichuris trichiura*-specific class-switched antibodies indicated prior exposure. No association was found between helminth-specific humoral responses and risk of *Mtb* infection, nor with maternal helminth-specific humoral responses. Conversely, data from murine experiments revealed a protective effect of maternal helminth infection (*Nippostrongylus brasiliensis)* on BCG infection in offspring, with reduced lung bacterial burden and increased numbers of activated $CD4^+$ T cells and B cells. Maternal *Nb* infection may have a synergistic effect on BCG vaccination, as BCG-vaccinated/infected pups from *Nb*-infected mothers had reduced lung bacterial burdens, increased $CD4^+$ T cell and B cell responses and increased IFNγ-producing $CD4^+$ T cells.

Findings from this study suggest that childhood vaccines could provide heterologous protection against unrelated pathogens such as *Mtb*. The murine data suggest a protective effect of maternal helminth infection against BCG infection in offspring, but no similar finding was observed with the clinical data. The clear protective effect of maternal *Nb* infection during offspring BCG infection warrants a more in-depth clinical study addressing the functional effects of maternal helminth infection on *Mtb* infection outcome in infants.

Chapter 1: Introduction

1.1 The infant immune system

A properly developed and functioning immune system is essential for our bodies to be able to respond to numerous internal and external factors that threaten our well-being. Underlying this system is the complex set of interactions that occurs between the two arms of the immune system, namely innate and adaptive immunity. Misplaced reactions or interactions within either arm of immunity gives rise to inappropriate responses to pathogens and other environmental stimuli, as well as auto-immune diseases. Although the response of the body to stimuli received after birth informs the maturation of the various immune cells, these responses would not be able to occur with inappropriate development of the immune system *in utero*. As such, it is important to consider how the various immune tissues, cells and mediators develop *in utero*, and then address immune maturation after birth and in the first years of life.

1.1.1 The two arms of immunity

1.1.1.1 Innate immunity

The innate immune system consists of the first frontier of cells that allergens or invading pathogens encounter upon gaining access to the body. These cells are able to respond to a broad array of allergens and molecules expressed by pathogens within the first few hours of detecting them. Although innate cells are classically understood as unable to respond with the same specificity as adaptive immune cells, their quick response provides robust initial control of an infection, allowing time for the adaptive immune system to be activated appropriately. **Figure 1.1** provides a brief overview of the innate immune cells.

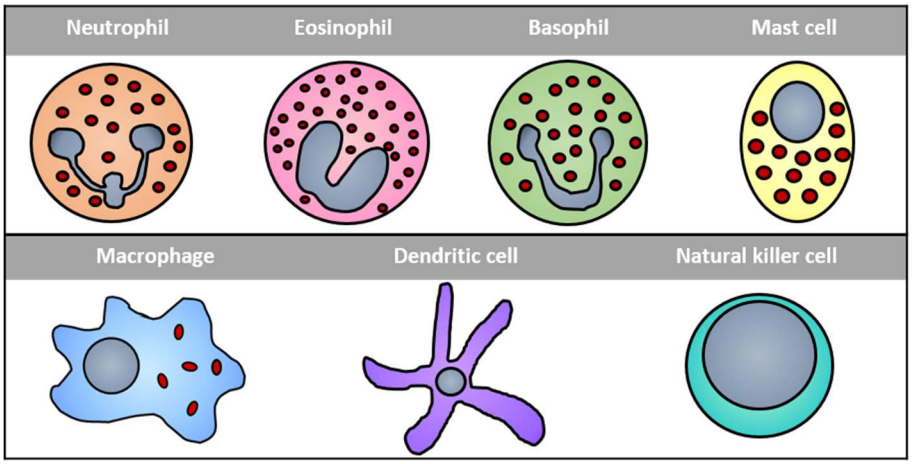

Figure 1.1. Innate immune cells (adapted from (1)).

Macrophages are phagocytic cells capable of killing invading pathogens and secreting molecules (cytokines, chemokines) to attract and activate other immune cells; macrophages, along with dendritic cells, are also responsible for presenting antigen to $CD4^+$ T cells to activate adaptive immune responses (1, 2). Neutrophils are granulocytes that aid in microbial killing, whereas eosinophils and basophils are the granulocytes involved in allergic responses and anti-parasite immunity (2, 3). Mast cells are mainly involved in allergic responses (2). Natural killer cells, innate cells derived from a lymphocyte precursor, are involved in anti-viral immunity and are able to kill antibody-coated cells via antibody-dependent cell cytotoxicity (4).

Despite the classical association of adaptive immunity alone with antigen specificity, more recent research investigating the roles of pattern recognition receptors (PRRs) found on innate immune cells has suggested this may not be wholly accurate. Toll-like receptors (TLRs) can detect pathogen-associated molecular patterns (PAMPs) from a wide array of micro-organisms (5), which is now known to occur through activation of either individual or combinations of TLRs (6). This stimulated cross-talk between TLRs, or between TLRs and other innate cell receptors, yields unique cytokine responses which can also be specific to a host cell type (7, 8). The inflammasome in monocytes and macrophages also requires activation through differential stimuli, either of TLRs alone or TLRs with an additional adenosine triphosphate (ATP) stimulus, which contributes to the specificity of responses by these cells (9). Nod1 and Nod2 receptors are able to detect distinct types of peptidoglycans in such a way that between these two receptors, all bacteria can be detected; these are also molecules which cannot be detected by TLR2 (10). Similar specificity has been observed with NK cells. A receptor present on certain NK cells is able to bind a particular MHC 1-like protein (thought to be a glycoprotein), resulting in resistance responses specifically to murine cytomegalovirus (11-13). Another level of specificity is added by the ability of NK cells to respond differently to this pathogen depending on its location within the host (14, 15).

Classically, innate immunity has not been associated with the generation of a memory response; however, thinking regarding this point is changing. Trained immunity is the observed occurrence that a primary infection can enhance protective responses to secondary infection, or to infection with an unrelated pathogen (16). It involves the PRRs, is less specific than adaptive immune memory, and it is suggested that epigenetic changes

underlie this phenomenon, at least in part (16, 17). Hallmarks of trained immunity have also been observed with vaccines, with certain vaccines (e.g. BCG) able to induce heterologous protection against unrelated pathogens (18, 19).

1.1.1.2 Adaptive immunity

The adaptive immune system consists of cells able to respond robustly to foreign particles or pathogens upon activation by specific antigens. Adaptive immune responses will only be initiated by the innate immune system if the pathogen cannot be controlled, and due to the activation process, adaptive immune cells will only contribute to protective responses at a later time-point, with the exact timing being dependent on the pathogen. **Figure 1.2** provides a brief overview of the adaptive immune cells.

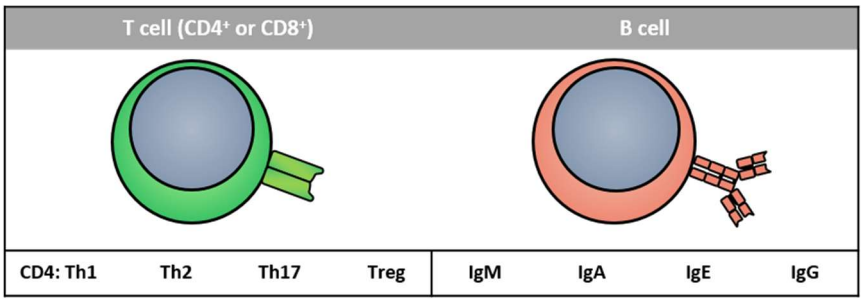

Figure 1.2. Adaptive immune cells (adapted from (1)).

Most T lymphocytes (T cells) are either $CD8^+$ T cells or $CD4^+$ T cells. $CD8^+$ T cells, otherwise known as cytotoxic T lymphocytes, are involved in anti-viral and anti-tumour immune responses (20). The $CD4^+$ T cells have more diverse functions which are dependent on their development and the cytokines by which they are stimulated. Broadly, $CD4^+$ T cells are classified as either Th cells or Treg cells. Upon stimulation by IL-12/IFNγ, $CD4^+$ T cells will polarise to a Th1 phenotype and begin producing IL-2, IFNγ and TNF-β (1, 21). Th1 cells help to activate macrophages and their anti-microbial defences, and they also aid in the activation of B cells and their antibody production; additionally, they are involved in $CD8^+$ T cell activation (21). Following stimulation by IL-4, $CD4^+$ T cells polarise to a Th2 phenotype and begin producing IL-4, IL-5, IL-6 and IL-13; these cells are involved in initiating the adaptive immune response to allergens and parasites, and they are also able to stimulate antibody production by B cells (1, 21). Stimulation of $CD4^+$ T cells by TGF-β and IL-6 yields Th17 cells which produce IL-6 and IL-17; these cells are involved in auto-immune responses (1, 22). Natural Treg cells develop as such in the thymus, whereas inducible Treg cells develop in the periphery in response to antigen stimulation (23). Both types express CD4, but are not Th cells. They are activated upon stimulation by TGF-β and produce TGF-β and

IL-10; they are responsible for the suppression of activation and effector functions of other T cells and are also involved in self-tolerance mechanisms (1, 24).

It is important to note that several classes of non-classical T cells have been identified, two of which will be discussed here. A small subset of T cells develops into γδ T cells as opposed to αβ T cells (classical T cells), and play a role in immunoregulation, immunoprotection and maintenance of tissue integrity (1, 25). These cells are unusual, due to their ability to detect antigens not recognized by classical T cells, such as non-protein or soluble protein antigens (1, 26). Additionally, their mode of antigen recognition is unique, as they do not require MHC binding (1, 25). NKT cells represent another non-classical T cell class. These cells express both NK and T cell markers and are reactive to CD1d, an MHC 1-like molecule (27). NKT cells are also known as good cytokine producers (27). These cells can respond directly to pathogens (e.g. Gram-negative bacteria, spirochetes) by recognizing unusual membrane glycolipid molecules, and indirectly by responding to cytokines produced by microbial DC activation (28). They have also been implicated in tumour immunology and protective anti-viral immune responses (27, 29).

B lymphocytes (B cells) represent the humoral component of adaptive immunity. They express MHC II which renders them able to act as antigen presenting cells for $CD4^+$ T cell activation, but their major function is antibody production (1). The activation signal received by the B cell determines which class of antibody is produced and the different antibody classes are located within different regions of the body: IgM is produced by mature naïve B cells (not yet class-switched) and is located throughout the body; IgA is produced by activated B cells and is found on mucosal surfaces and in breast milk; IgE is produced by activated B cells and is found on epithelial surfaces; IgG is also produced by activated B cells and is found throughout the body (1).

Adaptive immune cells also differentiate into memory cells during an immune response; these cells are able to circulate throughout the body and will be responsible for initiating a more rapid immune response should they ever encounter their cognate antigen again.

1.1.2 Development of the immune system *in utero*

Haematopoietic stem cells, the progenitors of all blood cells, can be detected from as early as the third week of gestation (30). At this early stage, these progenitor cells are produced by the yolk sac and chorion; from the second month of gestation the liver becomes the

primary haematopoietic organ, with its cell production only decreasing after the seventh month (30, 31). The thymus, which when fully developed is the site of T cell maturation, begins to develop by the end of the sixth week of gestation and becomes lymphopoietic by the end of the second month (30). The spleen is fully developed by week 8, with it and the bone marrow become haematopoietic by approximately weeks 10-11 of gestation (30, 32). Large numbers of these progenitor cells can be detected in the foetal circulation by 12 weeks (33).

Macrophages are the first of the differentiated leukocytes to be detected, and have been identified in foetal liver sections taken at 4.5 weeks old (34). At 4-6 weeks gestational age, cells within the mesenchyme and yolk sac, showing dendritic cell morphological features, have been identified as expressing monocyte- or macrophage-associated markers (35). These cells migrated from their tissues of origin and seeded various primary and secondary lymphoid organs (35). Eosinophils are produced in the foetal liver at detectable levels from week 5, with their numbers increasing gradually (36). Putative prothymocytes have been detected in the liver from week 7, with $TCR\beta^+$ cells identified in the liver from week 10 (37). T cell precursors have been detected in the developing thymus from week 8.5, with $TCR\beta^+$ cells increasing from week 9.5 to birth (38); $TCR\gamma\delta^+$ cells, although rare, could be detected in the thymus from week 10.5 (37). Pro- and pre-B cells can be found in both the liver and omentum by week 8, and are being produced in large numbers in the bone marrow by 16-20 weeks (39). Mature naïve B cells can be detected in the spleen from week 14, and it is suggested that these are generated in the liver prior to their maturation (40). A substantial proportion of these splenic B cells (measured in weeks 19-22) express CD5 (41), a marker of B1-B cells; these cells respond independently of T cells and can produce polyreactive antibodies (39). The antibodies IgG and IgM can be detected in the spleen by week 10, with their production peaking during weeks 17-18 (39). Serum IgG levels are observed to start increasing at an earlier time-point (5.5 weeks) and increased throughout gestation; however, it is important to note that maternal IgG is transferred to the infant via the placenta and constitutes most of the IgG present at birth (39, 42). IgE can be detected in both liver and lung by 11 weeks, and is observed in the spleen by week 21 (43). It is interesting to note that at 18 weeks gestational age the spleen contains all of the cells required for a robust immune response, and can take part in immune reactions from this point onwards (39, 44). Not only are mature leukocytes present in foetal lymphoid tissues,

but they can also be detected in the circulatory system. In a study of foetal blood samples taken *in utero* between 18 weeks and birth, the proportions of leukocyte sub-populations were measured to determine the normal levels of these cells at different points of foetal development (45). From 18-29 weeks gestational age, the following proportions were observed: lymphocytes comprised 85-88% of the cells present, neutrophils 6-8.5% and eosinophils 2-4% (45). From 30 weeks onwards, the proportion of lymphocytes decreased to 68.5%, and neutrophil and eosinophil proportions increased to 23% and 5% respectively; basophils were consistent from 18 weeks to birth (0.5%), as were monocytes (~3%) (45).

1.1.3 The immune system at birth

The robustness of the infant immune system at birth may have been under-appreciated previously, but it is clear that even though it may be naïve, the immune system at birth has largely completed its development. It is, however, important to note that differences in cellular function do exist within the infant immune system as compared to an adult's. Monocytes isolated from cord blood exhibited impaired chemotactic abilities, but normal phagocytic and killing capacity when compared to the activity of monocytes isolated from adult blood (46). It has also been observed that dendritic cells derived from cord blood have an impaired ability to stimulate T cell responses *in vitro* as compared to adult responses, with this impairment suggested to be due to their decreased expression of ICAM-1 (a cell-adhesion molecule) and MHC I/II (necessary for antigen presentation) (47). Mononuclear cells and macrophages isolated from cord blood exhibit impaired in cytokine production even with stimulation (48), with decreased levels of TNF-α (49) and IL-4 (50) detected and almost no IFN, even though cellular proliferation was not impaired (51). Natural killer cells, which fall within the grouping adaptive immune cells, also exhibited decreased cytotoxic activity at birth (52).

T cells have been the focus of numerous studies, due to their fundamental role of utilising innate immune signals to activate adaptive immunity when required. Several studies have investigated the T cell populations within cord blood in comparison to those populations observed in adult blood samples, to identify functional differences yielding either beneficial or detrimental effects. Researchers have observed that a large proportion of cord blood $CD4^+$ T cells have a naïve phenotype, expressing CD45RA, with fewer effector memory T cells detected (48, 53-55). These naïve T cells have been shown to exhibit decreased function and cannot be activated to the same extent as adult T cells (48, 56); they also

express lower levels of the activation markers IL-2 receptor and HLA-DR (54, 57). It has been observed that γδ T cells are more prevalent in neonates, and that they exhibit pleiotropic responses and IFNγ production (58, 59). This suggests that these cells are better positioned for early-life immune protection than the classical T cells described above (59). It is important to note that the developing infant's immune system has a significant immunosuppressive T cell component (60). The production of these Tregs is initiated during foetal development, and the process yields functionally capable cells able to suppress anti-maternal responses and the infant's own T cells (61, 62). The $CD4^+CD25^+$ Tregs detectable in cord blood are mature but naïve, and their proportions are comparable to adult levels (55).

At birth infants display selective antigen reactivity, with their antigen presenting capability developed enough to present alloantigen (39, 63). B cells found in cord blood can be classified as naïve but mature, as they can produce IgM, IgA and IgE if stimulated appropriately (53, 64). However, as neonates have yet to be exposed to external immune stimuli, their cord blood contains low levels of IgM, IgA and IgE, as well as memory B cells (39, 53). The IgG levels at birth can be higher than the levels observed in mothers, but most of the IgG detectable in cord blood is maternally-derived (39, 42). A closer analysis of the available information reveals that infants are born with close to fully functional innate and adaptive immunity, with the major limitation being a lack of antigen stimulation to induce immune maturation. Thus it can be seen that infants are able to initiate certain immune responses from birth, which allows them to combat the vast antigen exposure that is initiated at this timepoint.

1.1.4 Immune system development in early life

Although the infant immune system at birth is able to initiate immune reactions and provide a level of protection, functional differences do exist between immune cells at this age and those of older children and adults. Cord blood eosinophils and neutrophils express lower levels of the pattern recognition receptor Mac-1, even following stimulation (65), rendering these cells less able to respond to pathogens. Both polymorphonuclear and mononuclear phagocytic cells exhibit decreased chemotactic capability at birth as compared to adult cells (66). Chemotaxis within the mononuclear cells remains below the levels observed with adult cells even up until 10 years of age, whilst it remains low within the polymorphonuclear cells until 2 years of age (66). Dendritic cells derived from cord

blood monocytes produce decreased levels of IL-12 and neonatal antigen presenting cells, although able to stimulate cell proliferation, are less able to stimulate IL-10 and IFNγ production by mixed lymphocytes and purified allogeneic adult $CD4^+$ T cells (67, 68). This is accompanied by the finding that mononuclear cells isolated either from cord or peripheral blood shows impaired production of IL-12, a cytokine important in Th1 responses (69); this impairment was still observed in children of 5 and 12 years of age and was suggested to be due to a lack of dendritic cells or an impairment in their function (69). Cord blood-derived macrophages exhibit elevated levels of IL-27 gene expression as compared to adults, suggesting the presence of higher levels of this immunosuppressive cytokine in neonates (70). Neutralisation of this IL-27 resulted in an augmentation of the IFNγ response to the BCG vaccine (70), indicating the potential potency of immunological differences between infants and adults. Overall, neonatal innate immune cells produce lower levels of IFNγ and TNF-α (inflammatory), and higher levels of IL-1β, IL-23 (inflammatory cytokines), IL-6 (both pro- and anti-inflammatory) and IL-10 (anti-inflammatory) than observed in adults (71); these differences result in a Th2/Th17 immune bias in neonates (71), leaving infants less able to respond to intracellular pathogens which generally require a Th1 bias. When investigating blood leukocyte cytokine profiles more broadly, it has been noted that the production of pro- and anti-inflammatory cytokines increases slowly with age (72). An initial upregulation in the production of IL-6, IL-10, TNF-α and IFNγ to levels comparable to those observed in adult blood is no longer apparent by 2 months of age (72); however, cord blood and neonatal peripheral blood do exhibit higher IL-6/TNF-α ratios than observed in adults (73). Natural killer cells develop and mature rapidly after birth, with their activity reaching a level comparable to adult cell responses by 1-5 months of age, with their proportions in blood suggested to increase with age (52, 74).

In children followed up from birth to more than 36 months of age, differences in the ability of peripheral blood leukocytes to respond to antigen were observed. In children between 6-13 months, these cells exhibited a selective ability to respond to antigens and were unable to present alloantigen (63). From 13-26 months, the cells showed improved antigen responses and by more than 36 months of age the antigen responses were equivalent to those observed with adult cells (63). As previously mentioned, cord blood $CD4^+$ T cells are predominantly naïve. However as age increases, the proportion of naïve $CD4^+$ T cells in the blood decreases, accompanied by a concomitant increase in memory $CD4^+$ T cells (74-76).

Following stimulation, cord blood lymphocytes were able to produce IL-2 but not IFNγ (77). Whereas IL-2 levels at birth were comparable to those observed in adults, IFNγ levels only reached this point upwards of 3 years of age (77). As with $CD4^+$ T cells, most B cells are naïve at birth; from this point their numbers increase, peaking anywhere between 1 and 6 weeks, only to decrease as age increases further (78, 79). Although some research suggests no significant age-associated changes in the proportion of most B cell sub-populations (74), it is more likely that the changes are subtle. It has been noted that the proportion of $CD5^+$ B cells (B-1 B cells) decreases with age, as does the proportion of immature B cells (74, 78). Naïve and transitional B cells detected in peripheral blood also decrease with age, accompanied by an increase in IgG^+ (switched) and IgG^- (non-switched) memory B cells (79). By 1 year of age, IgG levels in infant blood have reached approximately 70% of adult IgG levels, but IgA only 30% (80).

The development of a child's immune system is not limited to any one age, but is rather something that occurs progressively over the first years of life. This insight is important when considering the potential factors that are able to influence this development, whether they be environmental, maternal or due to vaccination; their scope and influence should not be underestimated.

1.2 Factors contributing to altered immune responses in children

1.2.1 Transfer of maternal immunity to the infant

An infant receives a wide array of substances from the mother via both the placenta and breast milk, including minerals, nutrients and various immune components that aid in their protection against environmental factors in the first months or years of life (81). The trans-placental trafficking of maternal IgG provides the infant's initial source of protection after birth (82). This transport is facilitated by the FcRn, which was first identified in the neonatal rat intestine and subsequently in humans (83, 84). The FcRn is also responsible for the uptake of IgG from breast milk in neonatal rats (85); since the discovery of its expression on cells in the human foetal intestine, it has also been suggested that it is responsible for IgG uptake from human breast milk (86).

Breastfeeding is generally considered to be more protective than formula feeding. In low-middle income countries, breastfeeding is suggested to play a role in decreasing overall infant morbidity and mortality, especially due to diarrheal disease (infectious or not) (87).

In resource-rich countries, exclusive breastfeeding for at least the first 4-6 months is associated with protection against respiratory tract infections (including pneumonia) and a decrease in the severity of infections if acquired (88-90). Breastfeeding has even been associated with protection against the development of atopic disease in children (91). More detailed analysis of the composition of breast milk provides insight into its protective capabilities. Breast milk contains a substantial amount of secretory IgA (sIgA), with the exact composition of IgA antigen specificities being wholly dependent on the mileu of antigens to which the mother has been exposed (82). The abundance of sIgA reflects its importance to protection of the infant; it can opsonize and neutralize pathogens, prevent adherence of pathogens to host cells and even modulate inflammatory damage stimulated by other antibodies (82, 92). Leukocytes are also abundant in breast milk, especially in the colostrum, with phagocytic cells being the most prominent, followed by lymphocytes (82, 93). These phagocytes are fully functional, live cells that are able to kill opsonized pathogens (93). Smaller immune mediators have also been detected in breast milk. Both pro- and anti-inflammatory cytokines (such as TNF-α, IFNγ and IL-10) have been observed, in addition to several complement proteins (94, 95). Not to be neglected are the non-immune components that exhibit anti-bacterial activity, such as lactoferrin and lysozyme enzymes, both of which can inhibit bacterial growth (82). A more recent observation is the role of breastfeeding in the establishment of the infant microbiome. The large intestine microbiome of breast-fed infants is more commonly colonized by lactobacilli, bifidobacteria and bacteria from the *Ruminococcus* genus (96-98). Formula-fed infants do show higher microbial diversity at first, but once breast-fed infants start consuming solid foods, their gut microbial diversity also increases (97). These differences in initial microbiome colonization are significant; infants at a higher risk for developing atopic sensitization or those that went on to develop allergy showed higher levels of colonization by clostridia, and lower levels of colonization by bifidobacterial (99, 100). These differences could be detected prior to the development of any allergies, which suggests that the composition of the infant's microbiome plays a role in protection against/susceptibility to allergic diseases (99, 100). As previously mentioned, it has been suggested that breastfeeding aids in the protection against allergic disease; its influence on the microbiome may be a contributing factor to the protection that has been observed.

1.2.2 Nutrition and the infant immune system

In low-middle income and resource-rich countries, poor maternal nutrition during pregnancy is associated with low birth weight of infants (101). This is significant, as low birth weight is associated with increased risk of morbidity and mortality in infancy and early childhood, especially when socioeconomic disadvantage is involved (102). Higher numbers of deaths due to infectious diseases have been reported amongst adults born in times of food shortage (increased prevalence of maternal malnutrition), suggesting that early life stressors may still be able to impair immune responses years later (103). Impaired fetal growth has also been implicated in increased susceptibility to tuberculosis later on in life (104). Maternal malnutrition limits nutrient availability during fetal development, which could in turn alter the transfer of immunity to the infant (105). As our bodies perceive malnutrition as a stress factor, it results in increased production of the stress hormone cortisol, which could influence the development of the infant immune system if maternal levels are raised during pregnancy (105). Research investigating immune function in West African children has suggested that there is no measurable defect in certain immune responses in children born to mothers who had not received nutritional supplementation during pregnancy as compared to those who did (106). However, this could indicate that immune programming could be due to something other than early-life immune impairment, or that the impairment may only become visible later in life (106). It has been suggested that birth weight could affect an infant's response to vaccination. This is supported by research regarding administration of the typhoid Vi vaccine to adults or adolescents: in adults, responses to the vaccine correlated positively with birth weight, and malnourished adolescents who were also undernourished *in utero* were less able to produce an adequate humoral response to the vaccine (107, 108). This vaccine relies more heavily on B cell responses, suggesting that B cell development may be impaired during fetal growth retardation (107).

Micronutrient deficiency and its effect on the immune system is as important to consider as macronutrient deficiency, with compounds such as zinc, vitamin A and vitamin D known to play a role in the functioning of the immune system (109-112). Zinc acts as both an antioxidant and an anti-inflammatory agent and its deficiency has been noted in developing countries (113, 114). Zinc deficiency has been linked to immune dysfunction in mice and humans, with the development of innate and adaptive cells being affected; it has also been

implicated in increased susceptibility to different pathogens (110, 113, 114). It has been observed in mice that even mild zinc deficiency during pregnancy depresses immune function for extended periods of time (115). Due to the potential detrimental effects of zinc deficiency on immunity, studies investigating the effectiveness of maternal/early life supplementation have been done. Zinc supplementation during pregnancy appears to be beneficial to the infant's immune response and on morbidity due to infectious diseases, and supplementation of preschool children significantly lowered the incidence of lower respiratory tract infections (116, 117). Vitamin A is a compound involved in innate and adaptive immune function, aiding in the appropriate development of Th cells and B cells; its deficiency has been associated with alterations in the immune system, such as decreased Th2-directed humoral responses and mucosal barrier repair (111, 118). Vitamin A supplementation has already been employed during pregnancy and in young children. Its use during pregnancy and breastfeeding boosts the production of pro-inflammatory IFNγ, which would in turn boost responses to intracellular pathogens, perhaps even in infants (119). Administration of vitamin A to children is known to reduce morbidity and mortality due to various bacterial and viral infections, particularly mortality associated with measles infection (120, 121). Vitamin D uptake generally occurs through exposure to sunlight. Its receptor is expressed by various innate and adaptive immune cells, and its activation generally results in immunomodulatory effects (112). Knowledge regarding the effect of vitamin D deficiency on infectious disease susceptibility in children is limited, although more recent research has implicated vitamin D deficiency in childhood TB and predisposition to pneumonia (122, 123); in adults it is strongly associated with TB and influenza (112, 124). It has been shown that neonatal serum vitamin D levels correlate with maternal serum vitamin D levels, and that subclinical vitamin D levels in neonates along with non-exclusive breastfeeding result in a higher risk of severe acute lower respiratory tract infection in these children (125, 126). The association between maternal vitamin D levels and children's responses to early life stressors in strengthened by the finding that vitamin D supplementation during pregnancy was associated with a decreased risk of wheeze development in children (127, 128).

Undernutrition forms one arm of malnutrition, with overnutrition resulting in obesity forming the other. Obesity is a world-wide concern, with substantial proportions of both children and adults in resource-rich and low-middle income countries considered

overweight or obese (129). Obesity is often accompanied by a low level of chronic inflammation, and a substantial number of children and adolescents exhibit impairment of cell-mediated immunity, including decreased bactericidal activity of polymorphonuclear leukocytes (130, 131). Obesity has been implicated in susceptibility to nosocomial infections, but little information regarding its role in community-acquired infections is available; however, more recent research has shown an association with increased severity and risk of fatal outcome from an H1N1 influenza infection (132, 133). Maternal obesity during pregnancy has been shown to influence neonatal immunity, with cord blood exhibiting decreased numbers of eosinophils and $CD4^+$ Th cells, reduced phagocytic cells responses and an increase in certain pro-inflammatory cytokines (134). Maternal obesity has also been associated with an increased risk of children developing either wheeze or asthma but not atopic eczema or hayfever, indicating this susceptibility may or may not be due to altered immune responses (135-137).

1.2.3 Environmental effects on infant immunity

Once a child is born, he/she is immediately exposed to a barrage of environmental stimuli never before encountered, with numerous antigens to which the child's naïve immune system could respond. Therefore, it follows that the environment in which a child lives would have a profound impact on the development and responses of the immune system.

Some of the earliest stimuli encountered are allergens; these are non-pathogenic molecules which are generally inhaled and stimulate allergic immune responses in some individuals. The sensitization of children to common allergens is particularly prevalent in developed countries; for example, in the United Kingdom, North America and Sweden early life exposure to house dust mite antigen or birch antigen is associated with the development of atopic disease (including asthma) (138-140). Higher concentrations of the allergens of interest were associated with earlier wheezing episodes (in the case of asthma) or sensitization within the first 3 years of life (139, 140). A closer look at the immune response stimulated by sensitization to common allergens revealed higher levels of IL-5, IL-10 and IL-13, with a correlation between responses to birch antigen and atopic disease development being observed (138). Conversely, exposure to certain potential allergens during early childhood (potentially at a higher than normal level) can be protective. It has been shown and accepted that living on a farm, with exposure to animals and unprocessed cow's milk among other things, is associated with protection against atopic/nonatopic

asthma as well as other allergies (141). However, stimulation of allergic responses is not limited to environmental antigens, but can also be induced by dampness, atmospheric pollution and tobacco smoke. A questionnaire study of children living in rural areas of Sweden indicated that children living in damp houses with parents who smoked exhibited the highest level of bronchial hyperreactivity and allergic asthma; a study with young Canadian children also observed an association between wheezing or asthma diagnosis and environmental tobacco smoke and damp housing conditions (142, 143). The effects of tobacco smoke are not limited to the development of asthma; parental smoking is also associated with an increased risk of respiratory illness such as pneumonia, bronchitis or tracheitis within the first year of life, with maternal smoking regularly noted as having the most significant effect (144-146). As mentioned, pollution may also play a role; a study comparing respiratory variables (e.g. asthma) of children living in rural or polluted areas of Italy revealed higher frequencies of these variables in the polluted areas (147). This study also noted an increased risk of respiratory infection within the first 2 years of life with passive smoking, and an increased risk of developing asthma if the child's parents were smokers (147).

It has long been known that inadequate housing, including inadequate waste disposal, water supplies and poor ventilation, as well as overcrowding within these houses, leaves individuals more susceptible to health problems (e.g. infectious diseases). Due to the seriousness of this problem, the World Health Organization even released "Guidelines for Healthy Housing" in an attempt to rectify the situation (148). These problems occur most frequently in low-middle income countries, or in regions of countries where socioeconomic disadvantage is apparent. Several studies in Brazil have revealed that an increased risk of illness or death resulting from respiratory infection are associated with (amongst other things) crowding, low socioeconomic status and poor sanitation (149, 150). In Kenya, an increased risk of lower respiratory infection was positively associated with the number of siblings; a higher number of siblings would be indicative of children living in crowded conditions (151). The link between crowded living conditions and respiratory infection are not only a recent occurrence. Data gathered in the United Kingdom in the late 1940s also revealed that infants born to families of a lower social class and who lived in crowded conditions were at a higher risk of developing respiratory tract infections such as pneumonia (152). Overcrowded living conditions are not only associated with an increased

risk of respiratory infection; children in India with severe community-acquired pneumonia and living in such conditions required longer hospital stays and changes in their antibiotic treatment (153). Although overcrowding is detrimental in some cases, it appears beneficial in others. In several instances, it has been shown that either living in crowded conditions or having more siblings was associated with a decreased prevalence of asthma or atopic sensitization (149, 154, 155). The converse of this has also been shown; children in Bangladesh living in a household with fewer individuals exhibited a significantly higher prevalence of asthma (156).

In low- and middle-income settings across the world, there is a high burden of diarrheal disease due to inadequate water supply, sanitation and hand hygiene (157). Diarrheal disease has been a severe burden in low-middle income countries for a long time, often being caused by enteric pathogens in animal excrement, as well as contaminated food and water sources. In several low-middle income countries it has been noted that water and food utilized by families, including weaning food, were contaminated with bacteria at a level higher than is internationally considered “safe” (158-160); use of contaminated food or water regularly leads to diarrheal episodes. Inadequate water and sanitation do not only result in diarrheal disease, but also parasitic disease. An estimate of global disease burden due to inadequate water, sanitation and hygiene measures showed that diseases such as diarrhea, schistosomiasis, trichuriasis, ascariasis, and hookworm infection were responsible for the majority of deaths worldwide (1999-2000) (161). As concerning as this is, the occurrence of these diseases is largely preventable. It has been shown that exclusive breastfeeding (i.e. avoiding exposure of the infant to contaminated food and water) can provide significant levels of protection against diarrheal disease, even without proper sanitation; the only measure with a stronger effect than breastfeeding was access to clean water within the house (162). Meta-analyses of data from numerous countries have also shown that improved water, hygiene and sanitation measures significantly reduce the risk of and morbidity associated with diarrheal and parasitic diseases (163, 164). It was also noted that child mortality also fell drastically once appropriate water and sanitation measures had been implemented (163).

The environmental factors mentioned are not an exhaustive list; even so, it is evident that environmental factors have a profound impact on the developing immune system. In some

instances, the undue strain imposed by these stressors on the immune system may even result in longer lasting effects on the immune system than is currently known.

1.3 Childhood infection and vaccination

1.3.1 The importance of vaccination

The initial naivety and continual development of children's immune systems renders them more susceptible to infectious diseases. Although the global under-5 mortality has decreased significantly since 1990, diseases such as pneumonia, diarrhoea and malaria still pose a significant threat to infant health in the absence of appropriate health care interventions (165-167). The above-mentioned diseases, as well as measles, sepsis and meningitis cause severe morbidity and mortality in neonates and children less than 5 years old; the most significant illness burden is carried by low-middle income countries, especially in sub-Saharan Africa and South Asia (165, 168, 169). Acute respiratory infections, including pneumonia, are also commonly observed in children younger than 5 and are often caused by viruses; again, low-middle income countries carry the greatest burden of these infections (170).

Despite the continuing problem of these illnesses in children, the situation would be far more dire if not for the Expanded Programme on Immunisation implemented by the World Health Organisation in 1974 (171). This was established to widen the global administration of life-saving vaccines and the strategy has proved successful; global vaccination coverage is currently stable at 86% and 2-3 million deaths are prevented annually (171, 172). However, greater efforts are required to ensure continued success; increased coverage could prevent a further 1.5 million deaths, and 19.5 million infants globally still do not receive these vaccines (172). Most vaccines administered to children provide more than 70% protection against the causative agents of disease, with a substantial proportion providing upwards of 90% protection (173). The one exception is BCG, which is known to provide variable levels of protection (0-80% depending on the study and region investigated) (173). The higher prevalence of vaccine-preventable diseases in areas such as sub-Saharan Africa, where vaccine coverage is known to be lower, indicates their overall success (173).

Neither the importance of vaccination nor the severity of vaccine-preventable diseases should be underestimated. A decrease in vaccine coverage in developed countries has led

to outbreaks of diseases such as measles and pertussis (174-178). Pertussis outbreaks have been linked to both waning immunity and decreased vaccine coverage (175, 176), but measles outbreaks are almost solely attributable to decreased vaccine coverage, with a substantial influence of parental refusal to vaccinate children for nonmedical reasons (176-178). An important point to note is that refusal to vaccinate not only affects those individuals, but also those that cannot be vaccinated due to their age or medical reasons (177, 178). Mina and colleagues (179) demonstrate further why vaccine-preventable diseases should not be underestimated. They demonstrated that the immunomodulatory effect of measles infection (not vaccination) on the immune system was present for longer than originally thought (179). More significantly, this effect resulted in increased mortality due to unrelated infections acquired after measles; this effect can be avoided by vaccination (179).

1.3.2 T cell-dependent versus T cell-independent responses

The heterogeneity of pathogens targeted by the EPI necessitates unique immune responses to be elicited by these vaccines; as such, it is important to note the different paths of immunity stimulated by different antigen types. Antigens that are able to stimulate the adaptive immune response can be broadly classified as T cell-dependent or T cell-independent. T cell-dependent antigens are proteins or those expressed on cells/pathogens (180). These proteins need to be processed by antigen presenting cells and presented on MHC II molecules to $CD4^+$ T cells; once activated, these Th cells activate the correct antigen-specific B cells to proliferate and produce antibodies (180-182). A proportion of these activated B cells, located in germinal centres in the lymph nodes or spleen, will differentiate to form memory cells (182). T cell-dependent protein antigens are often those used in subunit vaccines. However, these proteins alone are unable to elicit protective immunity due to the lack of $CD4^+$ T cell stimulation by activated APCs (183). In the case of subunit vaccines, this prerequisite activation of the innate immune system is performed by adjuvants such as alum; modern adjuvants take into account the necessity of robust T cell responses for protection against certain diseases (e.g. TB) by including agonists for specific innate immune receptors (e.g. TLRs) (183, 184). T cell-independent antigens are categorised either as type 1 or type 2. Type 1 antigens are generally capsular polysaccharides linked to lipids (e.g. LPS); they bind to TLRs, and via TLR signalling they stimulate polyclonal B cell responses (180, 181). Type 2 antigens are polymers, e.g.

polysaccharides or polynucleotides; these molecules bind directly and specifically to the BCR and are able to induce memory B cell formation (180, 181). It is important to note that linking a T cell-independent polysaccharide to an immunogenic carrier protein can stimulate a T cell-dependent response, resulting in an earlier peak of antibody production and earlier protection; such a time difference is crucial when considering invasive bacterial infection in children (180).

Vaccines licensed for use in humans fall into 2 categories: live attenuated and subunit vaccines. As live attenuated vaccines contain whole pathogens, they would be able to stimulate either T cell-dependent or T cell-independent responses (or both), depending on the immunogenic antigen type. Subunit vaccines consisting of polysaccharide alone would stimulate T cell-independent response, whereas those containing protein or protein-linked polysaccharide would elicit T cell-dependent responses. Live attenuated vaccines are considered the gold standard, as they most accurately mimic natural infection and induce life-long immunity (185). Subunit vaccines, on the other hand, are generally adjuvanted to induce longer-lasting immunity, and are generally administered several times to boost and strengthen the primary response (185). Vaccines on the South African EPI that fall into the live attenuated category are oral polio, BCG, rotavirus and measles; those that fall into the subunit vaccine category are the pneumococcal conjugate vaccine, *Haemophilus influenzae* type B, hepatitis B, the tetanus and diphtheria toxoids and acellular pertussis. All of these vaccines are known to induce protective antibody responses, with these responses used as the correlates of protection against infection (186). Generally, the understanding has been that Th cell stimulation of B cells is required to generate protective and long-lived immunity to foreign antigens. However, it is important to note, especially in the context of the above-mentioned subunit vaccines, that T cell-independent antigens can induce the same effects in B cells (187-190).

1.3.3 The role of cell-mediated immunity in vaccine responses

As alluded to above, the success of most of the vaccines currently in use resides in their ability to induce protective antibody responses, and stimulate the formation of memory B cells that could target future infections; as noted above, these protective responses cover a wide array of pathogens (191). However, several diseases for which wholly successful vaccines have yet to be found require robust T cell responses for the pathogens to be cleared by the host.

It has been observed that numerous viruses which cause either acute or chronic infection (with vaccines licensed for some of them) require either an antibody response, a cell-mediated response or a combination of both for successful clearance (192). The influenza virus is an example of an infection that requires a combination of cellular and humoral immunity; although humoral immunity is essential in protection against the virus, cytolytic $CD8^+$ T cell responses are required for viral clearance once infection occurs (193). It has been documented that humoral responses wane in older individuals, and that the success of an influenza vaccine in these individuals is linked to robust T cell responses rather than the humoral response (194). Several types of influenza vaccines are available, but the introduction of a vaccine that is able to induce humoral and cellular immunity may provide longer lasting immunity, especially when used in combination with more commonly used vaccines able to induce robust humoral immunity (193). It is well-known that measles vaccination induces protective, long-lived humoral immune responses, but the role of cell-mediated immunity in response to vaccination should not be neglected. Although robust humoral immunity is required to prevent infection, both $CD4^+$ and $CD8^+$ T cells are required for viral clearance once infection has occurred, and for the establishment of the long-lived memory B cells (195). These T cells are also able to control a measles infection even in the absence of antibody (195). Administration of the measles vaccine has been delayed due to the interference of maternally-derived antibody; however, early administration (6 months) of the vaccine is able to prime measles-specific T cells (196-198). Although the response induced was not as robust as observed in adults (196), it was equivalent to cell responses measured in older children (197). Early administration of the vaccine may not induce a sufficient humoral response, but primed T cells could improve responses to booster vaccination (198).

The importance of cell-mediated responses in vaccine-induced immunity are not limited to viral infections. In a comparative study of children vaccinated with acellular pertussis and those not vaccinated (with/without history of infection), cell-mediated responses were observed more frequently in vaccinated children (199). Humoral responses are also required for pathogen clearance, and serum antibody to pertussis toxin was more readily detectable in vaccinated children and those with a history of infection (199). Humoral immunity to *Bordetella pertussis* is known to wane without booster vaccination; this is accompanied by waning of the cell-mediated immunity in some children (200). An

interesting observation is the increase of cell-mediated immunity in some children, which suggests that cell-mediated responses to this pathogen can be acquired naturally simply through exposure to the pathogen (200).

It is well-established that a robust T cell response (in both the CD4 and CD8 arms) is required for the control of *Mtb*, so it follows that a successful vaccine against *Mtb* would be able to induce long-lived memory T cell responses in addition to a humoral response (201). The only vaccine currently licensed for use against *Mtb* is *Mycobacterium bovis* bacille Calmette-Guérin, which is used to protect against disseminated forms of TB in infants; it's protection against pulmonary tuberculosis varies greatly and is dependent on age, location and exposure to environmental mycobacteria (202). Information on the mechanisms through which BCG affords protection is rare; however, a study investigating the immune response to BCG in young infants revealed its ability to induce robust $CD8^+$ T cell responses, and whole blood stimulation revealing high levels of IFNγ or IL-10 production (203). A recombinant BCG vaccine, designed to facilitate release of BCG into the cytosol and in turn activate $CD8^+$ T cells through MHC I, provided greater levels of protection against *Mtb* infection in mice as compared to standard BCG (204). Administration of an adjuvanted *Mtb* fusion protein (Mtb72F) was able to stimulate robust $CD8^+$ T cell responses and IFNγ production; it also provided a level of protection against virulent *Mtb* comparable to standard BCG (205). Antigen 85A, an immunogenic protein secreted by *Mtb*, when administered as part of a modified vaccinia virus Ankara vector, can stimulate the proliferation of antigen-specific T cells; it also boosts the levels of IFNγ-secreting antigen-specific T cells in BCG-vaccinated individuals (206). Despite the preliminary success of some novel TB vaccines, these findings do not always translate to the larger population (207). It should also be noted that although the strain of BCG used may not affect efficacy of the vaccine (202), the responses induced by the vaccine are strain-dependent (208).

It is apparent that the role of cell-mediated responses in immunity induced by vaccination should not be underestimated; rather, the potential benefits of designing vaccines to stimulate cell-mediated responses in addition to humoral responses should be considered.

1.4 *Mycobacterium tuberculosis* infection and active disease

Infection caused by *Mtb*, along with active tuberculosis disease represent one of the greatest global infectious disease burdens; cases in South Africa represent a substantial contribution to this burden **(Fig. 1.3)** (209). A major contributing factor to this high TB burden, is TB-HIV co-infection. In KwaZulu Natal, a province of South Africa, approximately 80% of individuals with active TB have HIV co-infections, and increased TB case fatality rates have been observed in populations with high HIV prevalence, e.g. sub-Saharan Africa (210, 211). Another point of concern in South Africa is the increasing incidence of drug-resistant tuberculosis, as treatment success rates and long-term outcomes for patients are poor (211, 212). Adding to this concern is the knowledge that, as previously mentioned, protection induced by BCG to *Mtb* infection and active TB disease is highly variable (202, 213-216). This highlights the importance of further drug and vaccine development if this disease is to be brought under control.

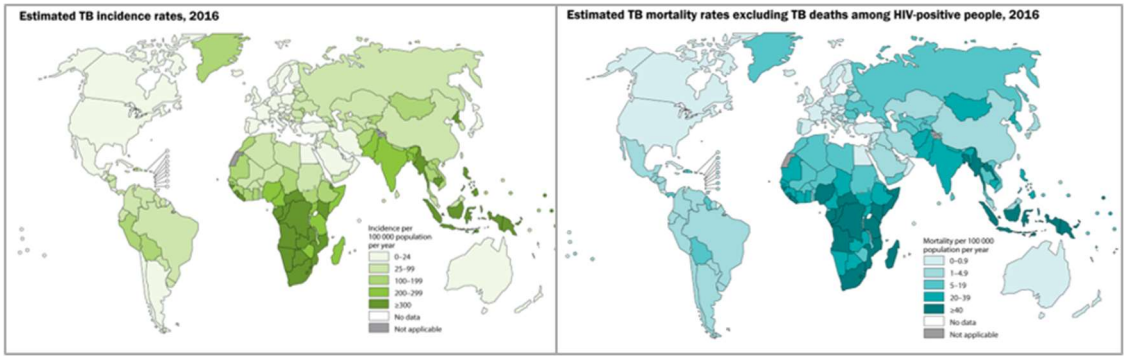

Figure 1.3. Estimated global TB incidence and mortality rates for 2016 (209).

1.4.1 The role of the immune system in *Mtb* infection and active TB disease

Upon inhalation and entry into the lung, *Mtb* bacteria infect and activate alveolar macrophages (217). Whilst some of the macrophage killing mechanisms remain functional and able to kill the bacteria, such as the activity of NOS2 (218), the ability of this pathogen to manipulate its environment to avoid killing has also been well-documented. One of the most well-known methods it employs is prevention of phagosomal maturation and phagolysosomal fusion (219-221). Experiments performed with *M. avium* revealed that although lysosomal protein LAMP-1 could still be identified on the phagosomal membrane, the protein required for acidification of the phagosome, ATPase, could not be detected (219). This is not to suggest that the cells are defective. Alveolar macrophages isolated from TB/HIV co-infected individuals were capable of phagocytosis and phagosomal acidification, but once the phagosomes contained *Mtb*, they were no longer able to acidify (222). The

delay in phagosomal maturation and decreased bacterial killing can be overcome by the induction of autophagy (223). However, it has been observed that phagolysosomal fusion in the presence of *Mtb* does occur in both dendritic cells and macrophages, but that the bacteria can subsequently escape into the cytosol (224). This process is *Mtb*-specific, requires the production of the virulence factors CFP-10 and ESAT-6 by the live bacteria, and induces death of the host cell (224). This induction of apoptosis is important to note, as alveolar macrophages isolated from individuals with active TB exhibit higher levels of apoptosis than those isolated from controls, suggesting this process as advantageous for bacterial survival (225). Another method the bacterium employs to survive within host macrophages, is to enhance superoxide burst and decrease lipid metabolism within the cells via TLR2/4 signalling, a process suggested to provide nutrients for the bacteria (221).

The establishment of an infection within the macrophages induces a host Th1 response largely directed against secreted antigens, with IFNγ and TNF-α identified as two of the important cytokines in this protective response (217). *Mtb* and BCG infection have been shown to induce the production of TNF-α, IL-12 (induces IFNγ production) and IFNγ by macrophages and dendritic cells (226-229). Additionally, dendritic cells exhibit upregulated MHC I expression following *Mtb* infection, which would be required for antigen presentation to $CD8^+$ T cells as the infection progresses (227). The absence of IL-12 leaves mice unable to control *Mtb* infection, and reduces T cells activation and granuloma formation (230). Conversely, IL-12 administered to susceptible mice reduces bacterial burdens and improves survival times; it is important to note that these protective effects also required the action of IFNγ (231). Disruption of TNF-α production or its action via its receptors is detrimental to survival in a murine model of *Mtb* infection, and interferes with control of the infection (232). The importance of TNF-α for the control of *Mtb* infection in humans has also been highlighted through the use of TNF-α antagonists (e.g. Infliximab) used in the treatment of certain autoimmune disorders (233, 234). It has been observed that such treatment is associated with an increased risk of developing TB, suggesting an important role for TNF-α in controlling *Mtb* infection in a latent state (233, 234). The effect of its absence on granuloma formation is not fully agreed upon, but its lack has been shown to induce necrosis, the starting point of bacterial dissemination (232, 235). The presence of fully functional macrophages and T cells ($CD4^+$ and $CD8^+$), but abnormal granuloma function in the absence of TNF-α, has been suggested as an indication of its role in granuloma

formation rather than cellular activation (235). Further support of this is the finding that TNF-α produced by human T cells stimulated macrophage aggregation, the first step in granuloma formation (236). The importance of IFNγ in control of *Mtb* infection was shown convincingly through studies of its disrupted function. In a murine model, mice unable to produce IFNγ rapidly succumb to infection; they are able to stimulate granuloma formation, but could not produce reactive nitrogen species and could not restrict bacterial growth (237). Similarly, individuals lacking IFNγ receptors either in full or in part, leave them more susceptible to mycobacterial infection, even with poorly pathogenic species, and decreases their macrophage TNF-α production (238, 239). Human cells have also been shown to produce the regulatory cytokine IL-10 in response to *Mtb* infection (236, 240). It has been implicated in the suppression of protective Th1 responses, as its neutralisation in PBMC samples obtained from TB patients resulted in increased levels of IFNγ production due to the presence of increased IL-12 (240). However, disruption of the IL-10 (or IL-4) gene in mice did not confer a greater level of protection against *Mtb* infection (241).

In murine models, both $CD4^+$ and $CD8^+$ T cells have been observed in lung tissue soon after *Mtb* infection, with their numbers peaking at week 8 (242-244). Both cell types are able to produce IFNγ in mice and humans, with human T cells also observed as TNF-α producers (236, 242-245). Protective murine IFNγ-secreting $CD4^+$ T cells are also able to recognise antigen presented by macrophages (242). Cytotoxic $CD8^+$ T lymphocytes can also be found in mouse lungs during *Mtb* infection, lysing macrophages via MHC I-dependent perforin expression (246). Additionally, T cells adoptively transferred from BCG-vaccinated mice to *Mtb*-infected mice conferred protection against the infection (247). Depletion of functional $CD4^+$ or $CD8^+$ T cells renders mice more susceptible to infection and causes necrosis within granulomas (248-250). It has been suggested that the loss of either cell type is associated with increased bacterial growth (249), or that a lack of control is only observed in the absence of $CD4^+$ T cells (and that $CD8^+$ T cell absence enhances control) (251). Whatever the immunological effect of $CD8^+$ T cell depletion, mice lacking $CD8^+$ T cells still succumb to *Mtb* infection earlier than wild-type mice (249, 251). Even though mice lacking $CD4^+$ T cells can control the initial infection due to recruitment of and IFNγ production by $CD8^+$ T cells and NK cells, $CD4^+$ T cells are suggested to be the cells generally responsible for early IFNγ production (250, 252). $CD4^+$ T cells have also been implicated in the normal formation of granulomas, a process crucial for long-term bacterial containment (252).

As alluded to already, the formation of granulomas to contain an *Mtb* infection represents an integral part of the protective host response. Initially, cytokines (as discussed above) activate macrophages and initiate granuloma formation within the lung (217). The bacteria are able to survive within macrophages, which aggregate at the centre of the granuloma; the remainder of the structure consists of dendritic cells, multinucleated giant cells, lymphocytes and fibroblasts (253-255). The lymphocytes (including $CD4^+$ T cells, $CD8^+$ T cells and B cells) form the outer layer of the granuloma, and a proportion of these cells will be closely associated with it **(Fig. 1.4)** (256). Intracellular bacteria contained within the granuloma are still able to recruit uninfected macrophages, which will phagocytose apoptotic infected cells and in so doing, aid in dissemination of the infection (257). Interestingly, it has been observed that whereas the centre of the human granuloma is considered pro-inflammatory (containing anti-microbial peptides and reactive oxygen species), the region immediately surrounding the central caseum is anti-inflammatory (258). Once the infection has been appropriately controlled as described, it enters latency, with bacteria able to survive for long periods of time within the granulomas. As infection progresses, the induction of Tregs and IL-4 has also been observed (217). Treg induction has been observed in individuals with active TB, and these individuals had higher numbers of these cells than healthy controls who responded to PPD testing (259, 260). These Tregs appear to contribute to the suppression of Th1 responses, as their depletion resulted in increased numbers of *Mtb*-specific IFNγ-producing T cells, and increased IFNγ production by T cells (259, 260).

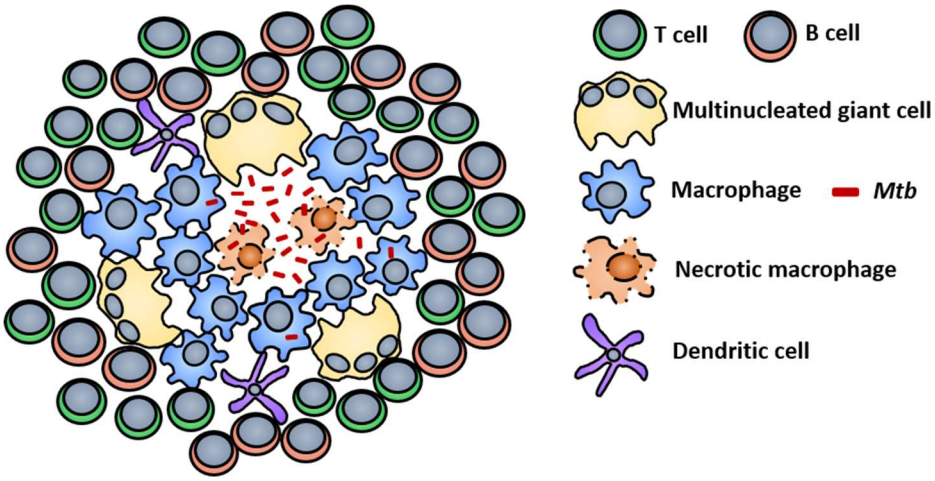

Figure 1.4. Schematic representation of a TB granuloma (adapted from (261) and (256).

1.4.2 Latent *Mtb* infection versus active TB disease

Even though an *Mtb* infection is often not eradicated, it can be controlled so effectively that only 5-15% of latently infected immunocompetent individuals will develop active TB disease during their lifetime (262, 263). However, any cause of dysregulation of the immune response controlling the infection (e.g. HIV infection) can cause reactivation, resulting in a productive infection that can be spread from caseating granulomas (255). Individuals that progress to active TB appear to have suppressed protective responses and increased regulatory responses. Patients with active TB, but that were unresponsive to PPD stimulation have IL-10-producing but not IFNγ-producing T cells following *in vitro* stimulation, whereas non-anergic patients with active TB had T cells producing both cytokines (264). The production of IFNγ by PBMCs from active TB patients (with or without HIV infection) is suppressed, as is proliferation of the PBMCs themselves; conversely, the production of regulatory cytokines TGF-β and/or IL-10 has been noted (265-268). Individuals that were followed up post-treatment revert to normal levels of TGF-β and IL-10 production, whereas IFNγ production remains depressed for at least 12 months (265). Neutralisation of these regulatory cytokines improves cellular proliferation, suggesting a role for these molecules in suppression of protective Th1 responses during active TB (267, 268). Increased levels of apoptosis have also been observed in patients with active TB, which could result in the loss of cells required for protection against *Mtb* (269, 270).

1.4.3 The role of effector and memory T cells in immunity to *Mtb*

Effector and memory T cells have an important role to play in secondary immune responses through both cytokine production and stimulation of other immune cells (271), resulting in a rapid response to the invading pathogen. As *Mtb* is an intracellular pathogen and T cell-mediated immunity is necessary for its control, it follows that memory T cells would be involved in long-term control of *Mtb*. In murine models the induction of long-lived, multifunctional memory CD4 T cells by vaccination has been observed, with these cells able to protect against subsequent *Mtb* challenge (272). Further research suggests that these may be central memory (CM) T cells (273). Additionally, memory CD4 T cells transferred to nude mice, which lack T cells, could confer protection against *Mtb* infection in these mice (274). Activated memory CD4 and CD8 T cells are able to initiate rapid IFNγ production in the lungs following a challenge infection, a finding not observed with primary infection (275); this is indicative of their importance in long-term mycobacterial control. In the

clinical setting, it is beneficial to consider the specificity, function and phenotype of memory cells isolated from latently infected individuals, as latency is associated with control of the pathogen. Regarding specificity, a genome-wide analysis of *Mtb*-specific epitopes recognised by memory Th1 cells from individuals with latent *Mtb* infection (LTBI) revealed large-scale specificity for antigens within bacterial secretion systems (276). Functionally, protective memory T cells are associated with the Th1 phenotype. Circulating PPD-responsive T cells isolated from healthy individuals were able to produce both IL-2 and IFNγ, with these observations remaining constant over a period of 6 months (277). An investigation of T cells in different stages of tuberculosis revealed that latent infection, minimal TB disease and decreased bacterial burden were associated with higher numbers of circulating ESAT-6-specific IFNγ-secreting CD4 T cells (278). Conversely, culture-positive active disease was associated with lower numbers of these cells (278). Several studies have investigated the phenotype of memory T cells detected during LTBI and active TB disease, but it remains unclear which memory T cells are most protective. Some research points to an association between CM CD4 T cells and LTBI, whereas active TB disease or risk factors for active disease may be associated with an effector memory (EM) CD4 T cell phenotype (279-281). This association suggests that CM CD4 T cells are the more protective phenotype (279, 280). However, this does not always appear to be the case. With CD8 T cells, the predominant phenotype is suggested to be EM, with differences in the differentiation state of these EM CD8 T cells being the feature associated with disease state (282). Additionally, a study of healthy individuals who had been treated for TB decades prior to investigation revealed the presence of long-lived, antigen-specific EM CD4 T cells, suggesting that in some cases, EM cells may play a role in protective responses against *Mtb* (283). It is also important to note that the protection they provide may not always be long-lived. In mice, it has been shown that EM and CM CD4 T cell responses induced by primary infection protect against the initial stages of *Mtb* re-infection, but that their numbers begin to wane, resulting in loss of pathogen control (284). These findings highlight the importance of memory T cells in protective responses to *Mtb*, which provide some guidance for vaccine design. However, the understanding of how to induce these cells to ensure protective functions and longevity is still not complete.

1.4.4 The role of B cell immunity in *Mtb* infection

The field of TB immunology has been dominated by elucidating the nature of protective cell-mediated responses, and the role of B cells in anti-*Mtb* responses has largely been neglected. However, there is increasing evidence suggesting the importance of humoral immunity in TB, and that antibody induction by vaccines may be beneficial (285). Murine studies have revealed a protective effect of passively administered *Mtb*-specific antibodies. Passive administration of a lipoarabinomannan (LAM)-specific IgG1 antibody affords protection against *Mtb* infection as indicated by reduced bacterial burden and weight loss, and increased survival (286). An arabinomannan (AM)-specific IgG3 antibody has also been shown to provide moderate protection against *Mtb* infection, causing bacteria to localise to the granuloma centre (287). Additionally, an IgA antibody specific for *Mtb* α-crystallin administered intranasally resulted in significantly reduced bacterial burdens (288). However, antibody protection is not limited to passive antibody administration. A BCG booster vaccine containing a combination of *Mtb*-specific antigens, Ag85A-ESAT-6, caused a reduction in bacterial burden and resulted in significant increases in serum immunoglobulin and bronchoalveolar lavage (BAL) IgA (289). The use of AM conjugate vaccines has also proven useful, with induction of specific IgG responses and protection against subsequent *Mtb* challenge (290, 291). It has also been shown that a lack of either inhibitory or stimulatory antibody receptors (FcRs) can influence *Mtb* infection. A lack of the inhibitory receptor FcγRIIB resulted in decreased bacterial burden and lung inflammation, but a lack of stimulatory FcγRs had the opposite effect (292). Studies utilising B cell knockout mice yielded contradictory findings regarding the role of B cell in TB. One study revealed that a lack of B cells resulted in increased bacterial burden and lung pathology (293), whereas a lack of B cells in early infection delayed bacterial dissemination and decreased lung inflammation (294). However, these findings may suggest differing roles for B cells at different stages of *Mtb* infection, and may depend on the strain of *Mtb* used for infectious challenge.

Several studies have reported on the presence of mycobacterial-specific antibodies and their potential role during infection and/or disease. BCG vaccination is known to induce antibody production, with IgG1, IgG2 and IgG3 considered the predominant subtypes (295); it has also been observed to induce AM- and LAM-specific IgG (296, 297). Numerous studies report raised levels of *Mtb*-specific antibody, often against AM or LAM and of various

subtypes (mainly IgG), in patients with LTBI or active TB disease as compared to healthy controls (298-303). As active TB disease occurs as a consequence of dysregulated immunity, the presence of raised *Mtb*-specific antibodies during active disease can be considered detrimental and may be involved in disease progression (303, 304). However, often *Mtb*-specific antibodies, whether from LTBI or active TB individuals, are suggested to be involved in the enhancement of protective cell-mediated responses. *Mtb*-specific antibodies have been associated with enhanced phagocytosis (300), phagosomal maturation (300, 305), classical complement activation (302), intracellular bacterial killing (305) and bacterial neutralisation (306). Additionally, it has been suggested that increased antigen-specific antibody could be associated with a reduced risk of developing TB disease, as children who developed TB disease exhibited significantly reduced IgG responses to Ag85A as compared to children who did not develop active disease (307).

The immune response induced by *Mtb* infection and active disease is complex and is not yet fully understood. A more complete understanding of the protective immune responses to *Mtb* would aid the control of this pandemic through improved drug and vaccine design, as well as through potential immunotherapy treatments. Additionally, the recent evidence suggesting an important role for humoral immunity in the protective immune response to *Mtb* warrants further investigation, as it could better direct the vaccine design approach.

1.4.5 *Mtb* infection and active TB disease in children

Children younger than 13 years were shown to contribute 13.7% of the total TB burden in a South African community (308), and a wider investigation of 22 high burden countries predicted the proportion of childhood TB contributing to total TB burden as 4-21%, depending on the country (309). Dodd *et al* also showed that, in 2015, approximately 240, 000 children younger than 15 years died of TB worldwide, with 80% of these deaths being children younger than 5 years (310). As such, TB falls within the top-ten causes of death in children worldwide (310). A primary *Mtb* infection in young children often progresses to serious disease within a year (311), which is accompanied by increased TB-associated mortality rates (312, 313). Young children generally do not present with bacteria in sputum samples (311), and are at the highest risk of developing disseminated forms of the disease (miliary TB, TB meningitis) (312, 314, 315). Children with acute TB disease have also been shown to exhibit impaired T cell function (316).

Several suggestions have been made as to the cause of altered and more severe disease in young children, with the consensus being that the immune system has not yet developed appropriately to combat *Mtb* successfully. It is known that dendritic cell immunity is under-developed in young children, with fewer cells detected in the circulatory system and downregulated IL-12 production observed (69, 317). Although chemotaxis is often downregulated (66, 318), the functional properties of monocytes, phagocytes and monocyte-derived macrophages in infants are similar to those of equivalent adult cells (319, 320). Conversely, neonatal alveolar macrophages exhibit impaired chemotaxis and reduced killing capacity (321, 322). It is also accepted that infant immunity is Th2-biased and that Th1 functionality is reduced (323, 324). This may have an influence on protective Th1 immunity to TB, as young children produce significantly lower amounts of IFNγ following T cell stimulation with mycobacterial antigens (325), and have an increased rate of indeterminate results with an *Mtb*-specific T cell IFNγ-release assay (326). Although such impairments could have a significant effect on an infant's ability to mount a protective response to *Mtb*, it is important to note that Th1 responses are not completely impaired, as BCG vaccination and exposure to a household TB contact can result in robust IFNγ responses (327, 328).

There is no doubt that *Mtb* infection and active TB disease in children, and the immune responses elicited are not fully understood. Considering the significant contribution of childhood TB to the global TB burden, further investigation of TB immunity in children is essential.

1.5 Soil-transmitted helminths

As with tuberculosis, soil-transmitted helminths are a global health concern, with approximately 2 billion individuals infected worldwide (329). Three of the most common infections are *with Ascaris lumbricoides* (human roundworm), *Trichuris trichiura* (human whipworm) and *Necator americanus*/*Ancylostoma duodenale* (human hookworms) (329). Importantly, ascariasis, trichuriasis and hookworm infection occurring in sub-Saharan Africa contribute substantially to the global burden of these diseases (330). Infections with these intestinal parasites are commonly encountered in children, and often exist as co-infections (331).

1.5.1 The helminth life-cycles and disease in humans

A. lumbricoides causes infection via the faecal-oral route (332). Following the ingestion of eggs, hatched larvae migrate to the lungs via the liver (332). Once in the lungs, larvae are coughed up and swallowed, after which they migrate into the small intestine where they develop into adult worms **(Fig. 1.5A)** (332). Infection with *A. lumbricoides* may result in disrupted nutrient processing and uptake, which could in turn contribute to malnutrition, especially in children (333). High infection levels often result in intestinal blockage in children, and via migration to biliary ducts or the appendix, the parasites may cause hepatobiliary/pancreatic ascariasis or appendicitis (334, 335). As such, a high helminth load often requires surgical intervention for parasite removal (334, 335). As mentioned above, *A. lumbricoides* larvae have a lung migratory phase. As they migrate through the lung tissue they may cause mild respiratory symptoms and eosinophilia, a condition known as Loeffler's Syndrome (336-338). The human hookworms *N. americanus* and *A. duodenale* have a lung migratory phase as part of their lifecycles. Infective hookworm larvae penetrate the skin and migrate to the lung once in the host's circulatory system (339). Larvae are then coughed up and swallowed, after which they migrate to the small intestine and develop into adult worms (339). These adult worms burrow into the intestinal wall to gain access to the host's circulatory system **(Fig. 1.5B)** (339). Hookworm infection has been shown to induce eosinophilia and gastrointestinal discomfort (340), and has also been implicated in stunted growth of school-age children (341). However, the most common morbidity associated with hookworm infection is anaemia resulting from intestinal blood loss, which can have far-reaching effects (339, 342, 343).

As with *A. lumbricoides*, *T. trichiura* eggs are ingested via the faecal-oral route, but unlike either the hookworms or roundworms, it does not have a lung migratory phase (331). Instead, once the eggs have been ingested and have hatched, larvae migrate to the colon, burrow into the epithelial lining and develop into adult worms **(Fig. 1.5C)** (331). Effects induced by infection with this parasite are not as well-known as with human roundworm or hookworm. However, it is known that moderate infection results in gastrointestinal symptoms that may amplify preexisting nutritional deficits, and heavy infection has been associated with anaemia (due to intestinal blood loss) as well as appendicitis and colitis (344). In some heavy infections *Trichuris* Dysentery Syndrome may occur, a condition

characterized by bloody diarrhea, rectal prolapse, anaemia, eosinophilia and growth retardation (345-347).

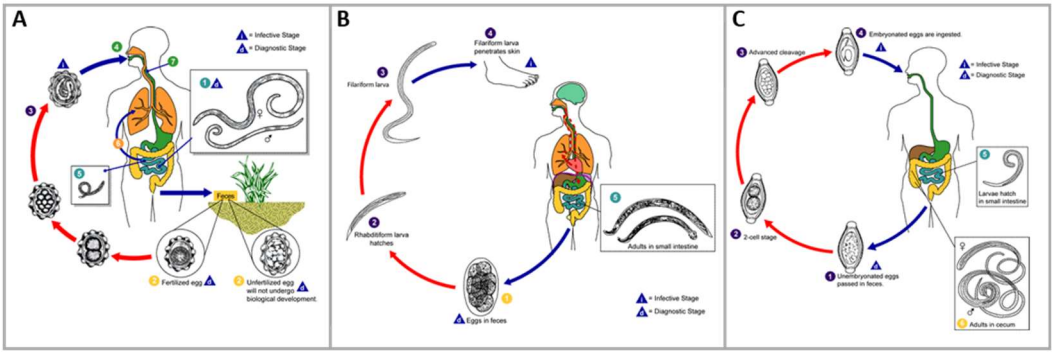

Figure 1.5. Life-cycles of human helminths *A. lumbricoides* **(A)**, human hookworm **(B)** and *T. trichiura* **(C)**. Adapted from the Centers for Disease Control and Prevention (https://www.cdc.gov/parasites/sth/).

1.5.2 Helminth-induced immunity

1.5.2.1 Innate immune responses

Please refer to **Figure 1.6** for an overview of the processes outlined below. The immune response to helminths has been well documented in various murine models of human helminth infection. As such, the detailed outline of helminth-induced immunity outlined below consists largely of data generated using these models. However, as will be discussed, a similar response has been observed with clinical studies. Helminths are known to induce potent Th2 responses, involving eosinophilia, Th2 cytokine production (e.g. IL-4, IL-5, IL-13) and Th2-associated class-switched antibodies (IgG1/IgE in mice, IgG4/IgE in humans) (348, 349). However, due to the complexity of these parasites, this Th2 response is complex and involves interplay between many immune and mechanical processes. Some of these processes will be outlined below, moving from innate to adaptive responses. Once a helminth parasite has entered the host, induction of alarmin (IL-25, IL-33) production is observed, often at mucosal sites of infection (350, 351). The presence of these alarmins, especially IL-33, activates innate lymphoid type 2 cells (ILC2s), recently identified cells that play a crucial role in Th2 cytokine production (IL-5 and IL-13) in early infection (350-353). This early cytokine production helps to direct the host response to the pathogen, aiding in the activation of innate and adaptive immune cells. It is well known that antigen presenting cells, such as dendritic cells (DCs) are necessary for the induction of CD4 T cell responses, but their exact role during helminth infection remains unclear. DCs have been shown to expand following helminth infection, but their phenotype and function appear to be modulated by the parasite (354). Research with mice resistant to *Trichuris muris* infection

has revealed a significant upregulation of chemokine production by intestinal epithelial cells, and that these molecules were responsible for DC recruitment to the intestine, whereas the same process was not observed in susceptible mice (355).

Irrespective of how they are activated, CD4 T cells are crucial in the host response to helminths, representing a primary cell type involved in Th2 cytokine production and parasite expulsion (348, 349, 356, 357). An important observation is that the robust induction of Th2 cytokine production is not redundant. Numerous studies have shown the necessity of these cytokines and the cellular pathways they activate in control of helminth infection, with particular importance ascribed to IL-4 for IgE production and IL-13 and the STAT6 cellular pathway for worm expulsion (358-364).

Various innate immune cells are induced downstream of the initial response and play different roles in the immune response to helminths. The induction of alternatively activated macrophages during helminth infection has been noted, but their precise function remains unclear. It has been suggested that their exact function depends on the helminth, with tissue repair, immune modulation and parasite expulsion named as some of these functions (365, 366). Two well-documented effects of helminth infection are an induction of mast cells (mastocytosis) and eosinophils (eosinophilia). Despite this, their precise roles during helminth infection are not well understood. *Trichinella spiralis* infection is known to induce intestinal mastocytosis, correlating with parasite expulsion (367). Mast cells have also been identified as the predominant IgE^+ cell type in the intestine following *T. spiralis* infection (368). Similarly, a lack of mast cells delayed expulsion of *Nippostrongylus brasiliensis* worms during a primary infection, but had no effect in subsequent infections (369). Conversely, the ablation of mast cells has no effect on *T. muris* infection, highlighting the uniqueness of responses to each parasite (370). As mentioned, eosinophilia is a hallmark of helminth infection, but the role of eosinophils during helminth infection is not fully understood (371). Migration of eosinophils towards helminths has been observed and they have been shown to contribute to protective responses in the lung during helminth infection (372, 373). However, eosinophil ablation does not render mice susceptible to helminth infection, suggesting a non-essential role for these cells during helminth infection (370, 373). Cell-mediated responses are not the only innate processes involved in control of helminth infection. Due to the sheer size of these parasites, several mechanical host responses are also required for successful infection clearance. Goblet cell

hyperplasia with subsequent increases in mucus production are necessary for parasite entrapment and expulsion, and are suggested to be under the control of IL-13 during helminth infection (363, 374). Particular attention has been paid to 2 mucin proteins, namely Muc2 and Muc5ac. Muc2 production is suggested to occur only in resistant mice, and its production is associated with worm expulsion and lowered worm energy status (375). Its importance is highlighted in Muc2-deficient mice, which exhibited significantly delayed worm expulsion (375). Muc5ac production is associated with IL-13 and its function is suggested to be essential for expulsion of *T. muris* and important for *N. brasiliensis* and *T. spiralis* expulsion (376). An additional consequence of *T. muris*-induced IL-13 production is an increased rate of epithelial cell turnover in the large intestine, aiding in parasite expulsion (377). Lastly, it is important to consider the role of smooth muscle contraction in parasite expulsion from the intestine. Again, the Th2 cytokine response drives this process, with intestinal muscle hypercontractility induced through IL-4/IL-13- mediated activation of the STAT6 cellular pathway during helminth infection (378). Deletion of IL-4Rα expression on smooth muscle cells, resulting in reduction of IL-4/IL-13 signalling, highlights the importance of this hypercontractility, as this deletion results in the downregulation of certain Th2 responses and delayed worm expulsion (379, 380). These important innate responses act in concert with adaptive cell responses (outlined below) to control and subsequently eliminate helminth parasites from the host.

1.5.2.2 B cell-associated immune responses

As already discussed, CD4 T cell are required for protection against helminth. The role of B cells, however, appears to be more complex. The Th2 response induced by helminths also induces B cell antibody class switching, favouring IgG1 and IgE production (381). Whether these antibodies are protective appear to depend on their antigen specificity and the helminth species that induced their production. *N. brasiliensis* infection does induce IgG1 production, but this did not relate to protective responses (358). However, a helminth haemoglobin-specific IgG1 antibody was able to reduce worm burden following *N. brasiliensis* infection (382), suggesting that antibodies specific for certain antigens may enhance protective responses to this parasite. Conversely, class-switched antibodies (IgG, IgG1, IgG2a/c, IgA) produced in response to *Heligmosomoides polygyrus* infection do play a role in protective responses, limiting egg production and worm development, and inducing larval trapping to reduce tissue damage (383-387). Similarly nuanced findings have

been reported for the role of IgE. In one instance it has been reported as important for immunity to *T. spiralis* (388), whereas another report suggests mice lacking IgE respond similarly to wild-type mice during *T. spiralis* or *N. brasiliensis* infection (389). However, the role of IgE may be more complex than previously thought. IgE (and IgG1) can induce basophilia in response to *H. polygyrus* infection (390), and the importance of basophils in protective immunity has been noted with the *N. brasiliensis* model (391). These findings suggest a role for IgE in basophil-mediated responses to helminths, a mechanism which warrants further investigation. Studies relating to overall B cell immunity have also been utilized to elucidate their role, with similarly mixed results. Total B cell deficiency does not appear to influence Th2 responses or worm expulsion following *N. brasiliensis* infection (392), but it has been observed that the B cell's antigen processing capability enhances protective responses to this parasite (358). On the other hand, the absence of B cells during *T. muris* or *H. polygyrus* infection is more noticeable, rendering mice susceptible (393) or delaying worm expulsion (392). Additionally, cytokine production by effector B cells was required to sustain the production of protective antibodies during *H. polygyrus* infection (383). Taken together, these studies highlight the importance of investigating both the humoral and non-humoral aspects of B cell function during helminth infection.

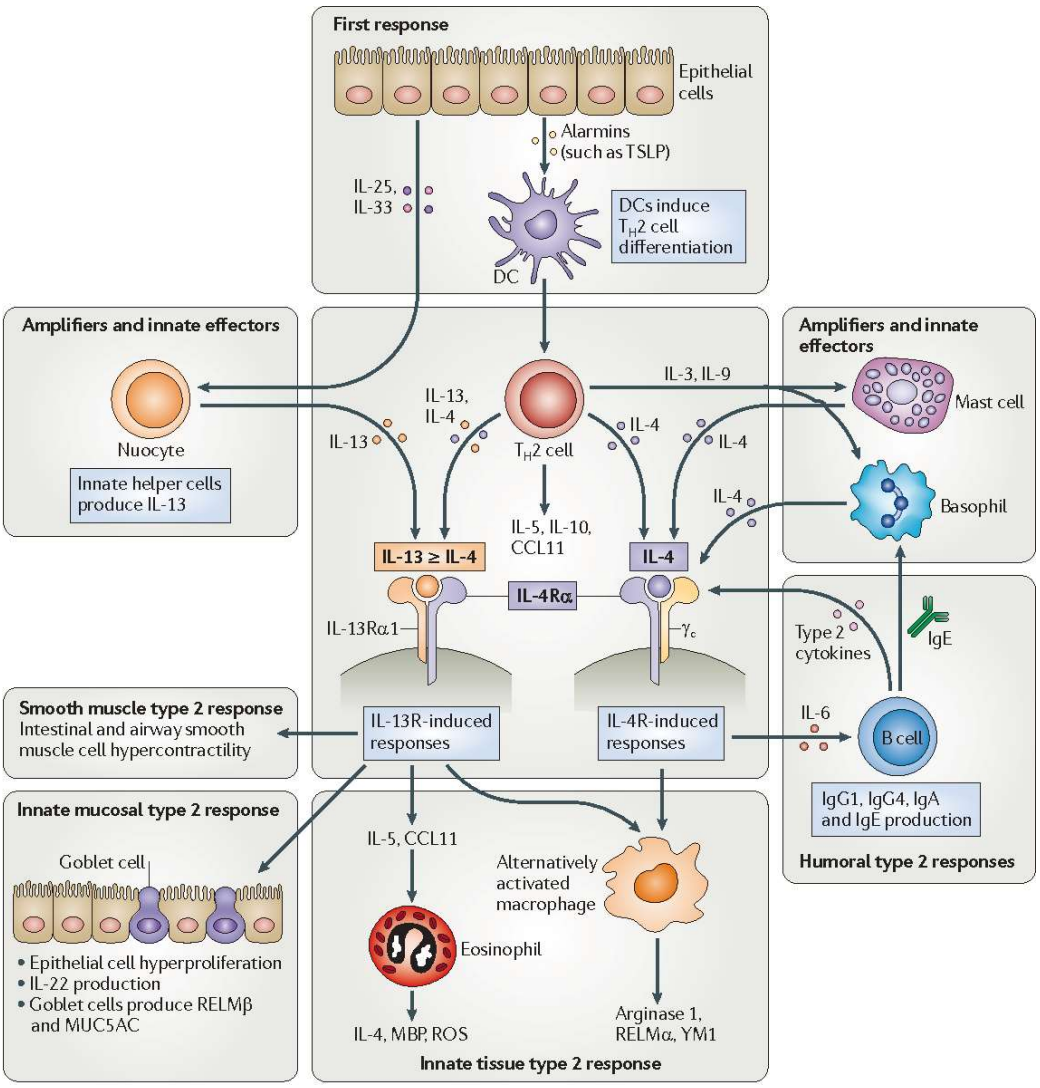

Figure 1.6. Overview of the major protective components of the type 2-associated response to helminth infection in mice (394).

1.5.2.3 Human anti-helminth immunity

Although research into the helminth-induced immune response is more challenging in the clinical setting, studies have revealed results that mirror observations from murine studies. Both *A. lumbricoides* and *T. trichiura* infection induce Th2 polarized cytokine responses (395-397), with robust Th2 cytokine responses associated with reduced infection intensity (398) and weaker responses with susceptibility to infection (399). Responses to hookworm infection are not as clear-cut. In one instance of experimental human hookworm infection, the helminth only elicited transient Th2 responses, with only eosinophil levels remaining elevated throughout the infection (400). Conversely, it has also been shown that experimental human hookworm infection can induce robust systemic and mucosal Th2 and regulatory cytokine responses (401). The difference in sample sizes utilized, and the innate variability of clinical studies may account for these reported differences, but the immune

response to human hookworm infection does require further study. Antibody responses in human helminth infection appear to follow similar patterns for different helminths, with IgE and IgG4 as the two most common antibody subtypes produced (402). The IgE subtype is most commonly associated with resistance to helminth infection and lower infection intensity, whereas IgG4 is associated with increased susceptibility to and higher intensity of infection (403-405). It has also been suggested that IgE and IgG4 antagonise each other's functions during infection (403), which would account for these observations. As with the cytokine responses, the antibody responses to human hookworm infection are not as simple. It has been suggested that IgM, IgG and IgE could provide protection against human hookworm infection (406), but it has also been suggested that despite elevated levels in adults, IgG does not appear to be involved in protective responses (407). Again, this highlights the necessity of further research into anti-hookworm immune responses in clinical studies.

Although the overarching protective response to helminths involves various pathways stimulated by Th2 immunity, each helminth (whether murine or human) elicits a unique combination of these mechanisms. Thus, despite their similarities, infection with any one or combination of parasites yields tailored immune responses to each parasite, rather than one broad response to all of them. This highlights the complexity of helminth immunology and how important it is not to overlook subtle differences in the responses induced, as such differences could have far-reaching effects on infection outcome.

1.6 Study aim and objectives

The above discussion reveals the pivotal role played by immune responses in disease outcome. These responses are especially important to consider and characterise in infants, a particularly vulnerable population group. The potentially important role that humoral immunity may play during mycobacterial and helminth infection has been mentioned, but its exact role is not yet fully understood, nor is the role of maternal immunity, despite it being one of the earliest immune influences an infant will encounter. These knowledge gaps are important to address, especially in the context of sub-Saharan Africa, a region with a substantial overlap of *Mtb* and helminth infection (330, 408) and often-limited access to appropriate healthcare. An improved understanding of the interplay between these infections, maternal immunity and childhood immune responses would be beneficial in the development of appropriate vaccines or treatments, which would be most useful to those

individuals living in regions endemic for these infections. The aim of my study was to investigate the role of humoral immunity and maternal infection history in infant's immune responses to vaccination and disease, with specific study objectives as outlined below:

1. *Characterise the overall plasma humoral response and compare the responses to Mtb infection.*
2. *Investigate plasma antibody responses to childhood vaccines and compare the responses to Mtb infection.*
3. *Investigate plasma antibody responses to helminths and compare the responses to Mtb infection.*
4. *Investigate the influence of maternal immunity on infant childhood immune responses.*
5. *Analyse the influence of maternal helminth infection on adaptive immune responses to BCG vaccination and infection in vivo.*

We hypothesise that anti-mycobacterial vaccination may induce antibody responses that afford some protection against mycobacterial infection, but that helminth infection (maternal or during early childhood) may interfere with these responses, or with responses to other childhood infections or vaccines.

Chapter 2: Investigating the role of humoral responses on immunity to *Mtb*

2.1 Introduction

It is well known that cell-mediated immunity is essential to the host's protective response against *Mtb*, due to its intracellular nature. For this reason, the ability of novel TB vaccines to induce cell-mediated immune responses is considered most important for vaccine efficacy (409, 410), despite the observation that even when a vaccine is able to induce robust cellular responses, this immunogenicity does not necessarily equate to a noticeable protective effect (207, 411, 412).

On the contrary, most other clinically efficacious vaccines are considered successful due to their induction of protective antibody responses (285, 413, 414), suggesting that induction of humoral immunity in addition to cell-mediated immunity by a TB vaccine could be beneficial, rather than essential. However, the induction of heterogeneous antibody responses by *Mtb* complicates the identification of antigens that would elicit sufficient protective antibodies in the context of vaccination (299, 415). Despite this difficulty there is promising evidence from murine and clinical studies that antibodies elicited by BCG vaccination or *Mtb* infection could enhance host protective responses (287, 292, 296, 297, 300, 307, 416).

Although several studies have investigated antibody production and the role of antibodies in *Mtb* infection/TB disease, a comprehensive understanding of their function is lacking. Overall, antibodies are suggested to enhance cell-mediated protective mechanisms. Opsonisation of *Mtb* has been shown to enhance bacterial uptake by macrophages (296, 297, 300), whereas neutralization and antibody-dependent cell-mediated cytotoxicity (ADCC) can inhibit bacterial growth (296, 297, 305). Subtleties such as variations in antibody glycosylation can also affect antibody-mediated bacterial control (305). In the case of active TB disease, it has been suggested that antibody isotype is important to consider, as IgA has been shown to enhance and IgG impair control of disease (415). Despite these promising findings, it is important to take into consideration that the complicated nature of *Mtb* infection and TB disease will likely translate into different roles for antibodies at various disease time-points.

Host vaccination status may also influence immune responses to *Mtb*. It has been observed that measles, BCG and tetanus toxoid-containing vaccines have differing effects on all-cause mortality (417, 418), and measles vaccination has been suggested to dampen the reaction to tuberculin, used to detect *Mtb* infection (419, 420). Despite these suggested heterologous effects of certain childhood vaccines, a closer analysis of their potential effects on *Mtb* infection is still required.

I sought to investigate whether plasma antibody levels in infants from a TB-endemic setting associated with risk of acquiring an *Mtb* infection. To achieve this, total IgG and antigen-specific IgG responses to related and unrelated antigens were measured and analysed in light of *Mtb* infection outcome.

2.2 Materials and methods

2.2.1 Cohort recruitment

Study participants

This is a sub-study of a double-blind, placebo-controlled efficacy trial of the MVA85A vaccine candidate, performed in the Western Cape Province of South Africa, and described previously (207). Infants who were 4-6 months of age and had received the BCG vaccine within 7 days of birth, were enrolled. Study inclusion criteria at baseline were a negative HIV ELISA result, a negative QuantiFERON-TB Gold In-tube (QFT) test (to detect *Mtb* infection by measuring IFNγ responses to specific mycobacterial proteins) and no known exposure to an adult with active TB disease. At the point of enrolment, infants must have received all routine childhood vaccinations as per the EPI. The parent trial showed that MVA85A boost vaccination did not afford more protection than BCG vaccination alone in newborns (207). Therefore, placebo (Candin®: *Candida* antigen) and MVA85A vaccinated infants were combined in the same analyses. One hundred and thirty-six infants were sequentially recruited into the sub-study at the time of TB investigation **(Fig. 2.1A)**. However, complete clinical data and sample sets were not collected for thirteen participants, leaving clinical data and samples from one hundred and twenty-three participants available for analysis in this study **(Fig. 2.1A)**.

The sub-study and parent trial (C-020-485) were both approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (study numbers

032/2010 and 291/2008 respectively), and written informed consent was obtained from the infants' parents or legal guardians prior to participation.

Cohort outline

Following recruitment at baseline, infants were followed up at 3-monthly intervals to identify symptoms consistent with TB disease. Identification of such symptoms triggered admission to the case verification ward for standardized investigation and QFT re-testing; such a visit is subsequently referred to as a TB investigation visit. Asymptomatic infants were admitted if an individual with TB disease became a new household contact (207). Where available, two plasma samples were analysed per infant for this sub-study: one taken from QFT testing at baseline, and one from QFT re-testing at a TB investigation visit **(Fig. 2.1B)**. Where multiple TB investigations occurred for an infant, analysis was limited to one TB investigation visit sample per infant. *Mtb* infection was defined by a single positive QFT test (interferon-gamma release assay conversion). Infants who were QFT positive, but did not show evidence of probable or definite TB disease (421) were classified as *Mtb*-infected. None of the infants, irrespective of QFT status, were diagnosed with active TB disease. Infants were stratified by QFT negative (*Mtb*-uninfected) and QFT positive (*Mtb*-infected) status in the subsequent analyses.

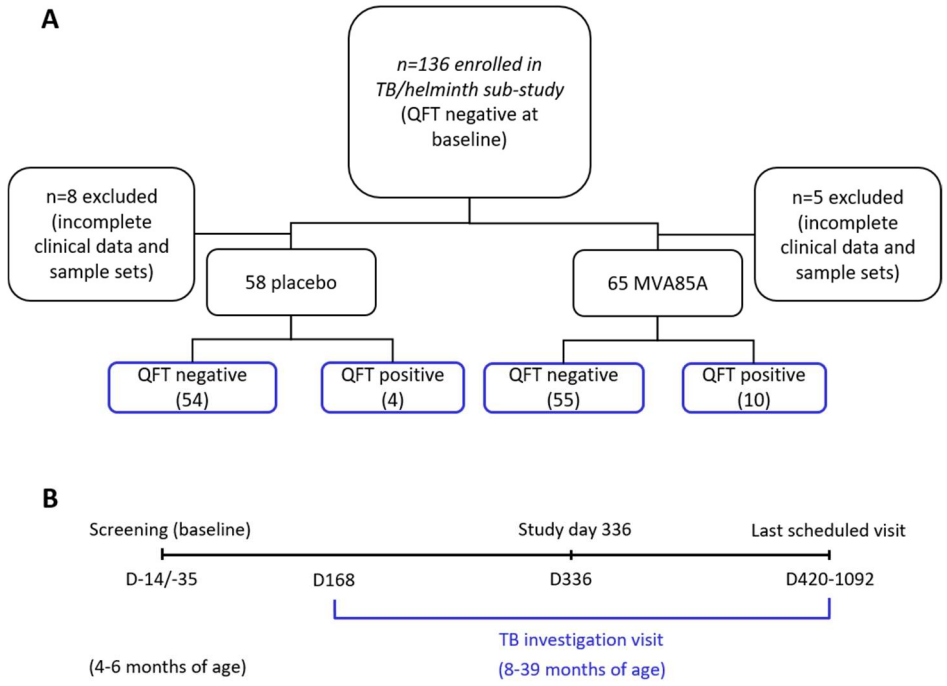

Figure 2.1. Infants recruited to the TB/helminth study. Infants enrolled in the MVA85A trial were randomly assigned to the placebo or MVA85A vaccination arms **(A)**. One hundred and thirty six infants were recruited to the TB/helminth study; incomplete clinical data and sample sets were available for n=13 infants, leaving n=123 available for further analysis.

Time-points of blood collection for QuantiFERON (QFT) **(B)** were at baseline, study day 336, at last scheduled visit and/or upon TB investigation. Per participant, serum was analysed at baseline and upon TB investigation.

2.2.2 ELISA

The concentrations at which all antigens and antibodies were used, were optimised specifically for use with this in-house ELISA assay. The procedures outlined below were adapted from similar methods described elsewhere (298, 299, 415, 422, 423).

Vaccine antigens

BCG (Danish strain 1331, Statens Serum Institut, Denmark), live-attenuated measles (Rouvax, Sanofi Pasteur) and tetanus toxoid (Tetavax, Sanofi Pasteur) vaccines were reconstituted in and diluted with 1X PBS to yield antigen stocks of 500µg/ml; aliquots of stocks were stored at -80°C until required.

Non-specific and antigen-specific ELISA

Both total IgG and antigen-specific antibody ELISAs were performed. Coating antigens were used at 5µg/ml for antigen-specific IgG or 10µg/ml for antigen-specific subtypes of IgG. Initial plasma sample dilutions were 1:50 (total IgG) and 1:20 (antigen-specific), followed by serial 1:5 dilutions of the initial dilutions across a further 7 wells. Primary and detection antibodies were used as described **(Table 2.1)**; all detection antibodies were alkaline phosphatase-linked.

Table 2.1. ELISA antibodies.

Antibody	Primary/ detection	Dilution	Clone	Company
Goat α-human IgG (Fc-specific)	Primary	1:5000	-	Sigma-Aldrich
Mouse α-human IgG (Fc)-AP	Detection	1:1000	JDC-10	Southern Biotech
Mouse α-human IgG1 (hinge)-AP	Detection	1:500	4E3	Southern Biotech
Mouse α-human IgG2 (Fc)-AP	Detection	1:500	31-7-4	Southern Biotech
Mouse α-human IgG3-AP	Detection	1:500	HP6050	Southern Biotech

Nunc-Immuno^{TM} MicroWell^{TM} Maxisorp^{TM} 96-well plates (Thermo Fisher Scientific; Waltham, MA) were coated with primary antibody or antigen and incubated at 37°C for 3 hours. Plates were washed 3 times with PBS-Tween20 buffer and blocked with 200µl/well 2% milk powder/1X PBS at 4°C overnight. Plates were washed 3 times as above and appropriately diluted plasma sample added (50 µl/well); plates were incubated at 4°C overnight. Plates were washed 3 times as above and detection antibody added (50 µl/well); plates were incubated at 37°C for 3 hours. Plates were washed 4 times as above and PNP substrate (Sigma-Aldrich; St. Louis, MO) added. Reactions were stopped with 1M NaOH and

plates read on a Versamax™ 96-well plate reader (Molecular Devices; Sunnyvale, CA) at wavelength 405nm (492nm reference filter). Arbitrary antibody responses were recorded from sample titration curves as the dilution at which a defined OD value was reached (423). Subsequently, these values were log transformed prior to further analysis to normalise the data, enabling a relative assessment of antibody levels in serum (423) **(Fig. 2.2)**. According to this analysis, antibody levels in certain samples and for certain antibody types fell below the detection limit and are observed at zero on the y-axes. This does not preclude the possibility of specific antibody responses within those samples, but may require detection with a more sensitive assay.

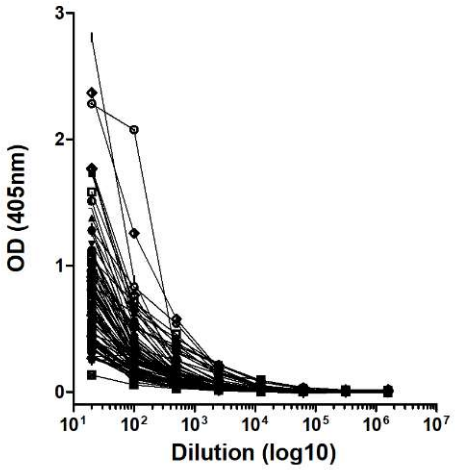

Figure 2.2. Sample titration curve. Example of sample titration curve generated from ELISA readouts and used to determine arbitrary antibody titres.

2.2.3 Statistical analysis

Dot plot graphs are represented with the median and interquartile range where appropriate. Antibody responses in matched sample pairs were compared using the Wilcoxon matched-pairs signed rank test, and the Mann Whitney test was utilized for the analysis of unpaired two-group data. All grouped analyses were investigated by the Kruskal-Wallis test and correlations were investigated using the Spearman correlation test; lines-of-best-fit are overlaid on correlation graphs. The Fisher's exact test was utilized to compare group frequencies as required. Where appropriate, analyses were two-tailed and significance was accepted at $p \leq 0.05$. GraphPad Prism software (v. 5.03) was used for all statistical analyses and graphs presented.

2.3 Results

2.3.1 Cohort description

Of the 123 infants, plasma samples were available for 100 infants at baseline (recruitment), whereas at TB investigation plasma samples and QFT status were available for all infants. The median age of infants at TB investigation was 19.5 months (interquartile range 15.5-25 months). Of the 123 infants, 71 (57.72%) exhibited TB symptoms, and 14 (11.38%) exhibited QFT conversion upon re-testing at TB investigation **(Table 2.2)**.

Table 2.2. Infant socio-demographic and clinical characteristics at TB investigation.

	N=123
Infant characteristics	
Age – months (median (IQR))	19.5 (15.5-25)
Gender (male)	53 (43.09%)
Weight at admission – kg (median (IQR))	9.23 (7.89-10.32)
TB symptoms (weight loss, failure-to-thrive, cough > 2 weeks) (YES)	71 (57.72%)
Positive QuantiFERON	14 (11.38%)

2.3.2 Infants exhibit an age-related increase in total IgG titres from baseline to time of TB investigation

Baseline and TB investigation plasma samples were analysed for total IgG levels. Pairwise analysis of the samples revealed a significant increase in total IgG titres from baseline to TB investigation **(Fig. 2.3A)**; titres measure at TB investigation also reveal a bimodal distribution approximately around the median. Further analysis revealed a significant inverse correlation between total IgG titres and age at TB investigation, indicating that older infants exhibited lower levels of total IgG **(Fig. 2.3B)**. Stratification of infants by QFT status showed that QFT+ infants were significantly older than QFT- infants (median age in months 28.5 vs. 19 respectively; p=0.002) **(Fig. 2.3C)**.

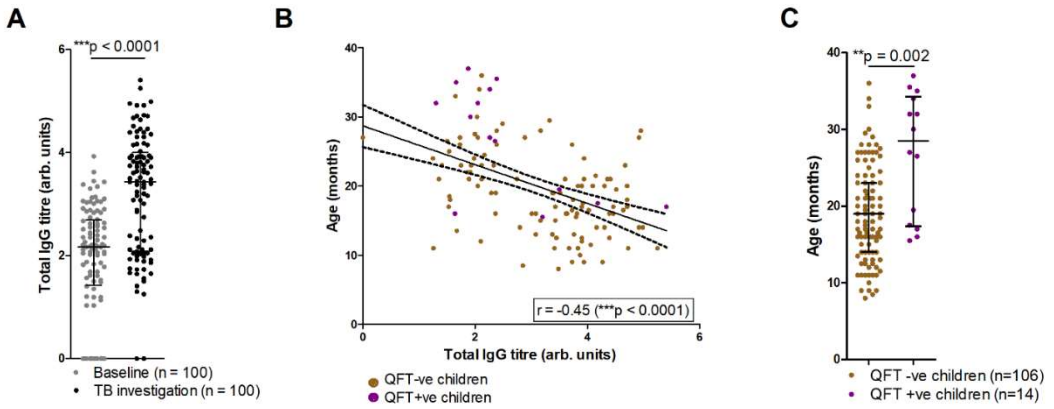

Figure 2.3. Infants exhibit an age-related increase in total IgG from baseline to TB investigation. Total plasma IgG in participants at baseline and upon TB investigation visit (n=100) as measured by ELISA **(A)**. Total IgG titres vs. age in months at TB investigation (n=120), with samples from QFT positive participants indicated in purple **(B)**; overlaid are the line-of-best-fit and 95% confidence bands (dashed lines). Association between age at TB investigation and QFT result **(C)**. Antibody titres are presented as log-transformed arbitrary values. Comparison in **(A)** was assessed for significance by the Wilcoxon matched-pairs signed rank test; comparison in **(C)** was assessed for significance by the Mann Whitney test. Strength of correlation in **(B)** was assessed by the Spearman correlation.

2.3.3 Total IgG titres increase significantly in QFT negative infants

As previously mentioned, QFT+ infants were older, but older infants also exhibited decreased total IgG titres. To investigate the potential role of total IgG in these associations, total IgG titres at baseline and TB investigation were stratified according to the infant's QFT outcome at TB investigation **(Fig. 2.4A)**. At baseline, there was no difference in median total IgG titre between infants who remained QFT- and those that later acquired an *Mtb* infection (QFT+) **(Fig. 2.4A)**, whereas at TB investigation, QFT+ infants exhibited a trend for decreased total IgG titres (p = 0.052), suggesting an association between higher total IgG levels and reduced risk of acquiring an *Mtb* infection **(Fig. 2.4A)**. Longitudinal analysis of individual infant IgG responses revealed a significant increase in total IgG titres from baseline to TB investigation in QFT-, but not QFT+, infants **(Fig. 2.4B)**. To investigate this association quantitatively, total IgG titres were compared to IFNγ concentrations measured by QFT. A significant inverse association was found between total IgG titres and IFNγ levels as measured at TB investigation **(Fig. 2.4C)**.

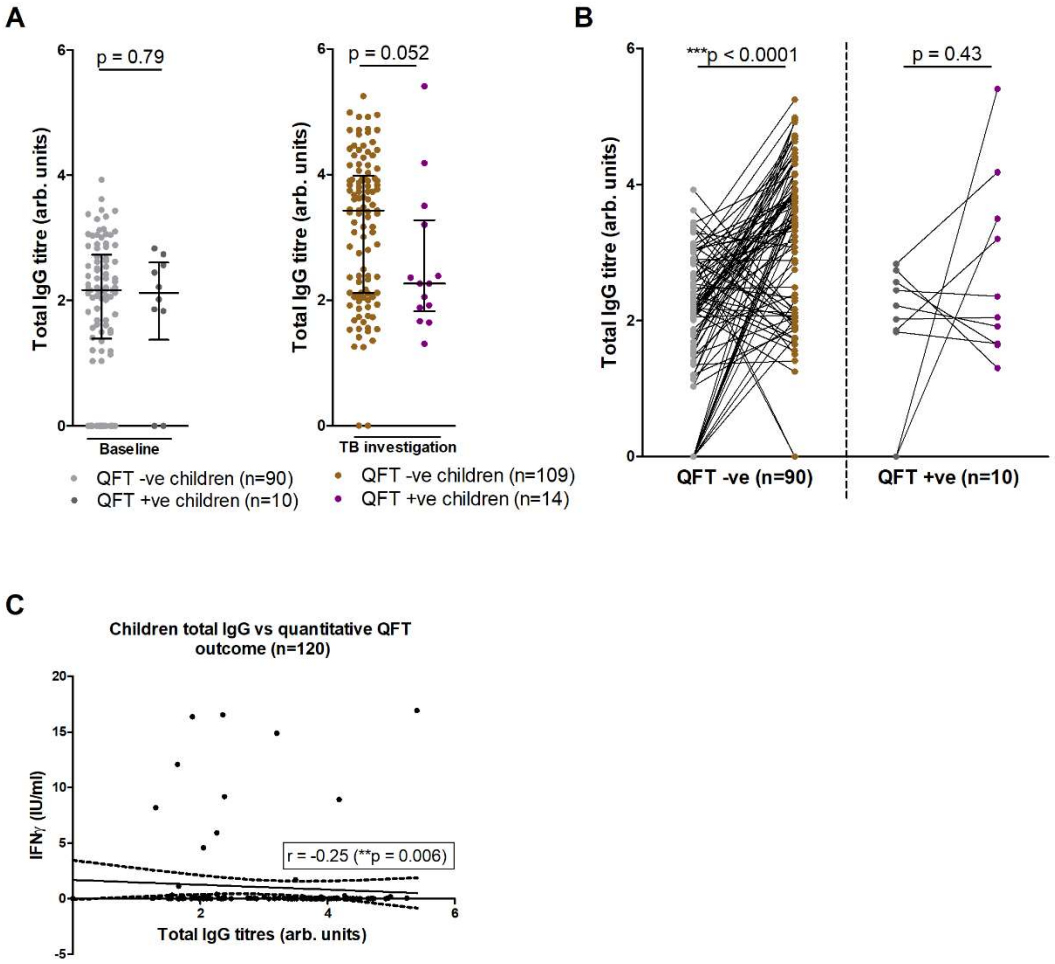

Figure 2.4. QFT negative infants exhibit a significant increase in total IgG titres. Relationship between QFT outcome and total IgG titres from baseline and TB investigation participant samples **(A)**. **(B)** is a before/after comparison of samples subdivided as in **(A)**. Total IgG responses vs. quantitative QFT outcomes **(C)**; overlaid is the line-of-best-fit and the 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Associations in **(A)** and **(B)** were assessed for significance by the Mann Whitney test. Strength of correlation in **(C)** was assessed by the Spearman Correlation.

As infants were recruited from the parent MVA85A vaccine trial cohort, the potential influence of vaccination with MVA85A or placebo (Candin®) on total IgG responses and QFT outcome was investigated. Similar frequencies of QFT+ outcomes were observed between infants receiving placebo or MVA85A vaccination, both at baseline and TB investigation **(Fig. 2.5)**. No differences between placebo treatment and MVA85A vaccination were observed when infant total IgG responses were stratified according to vaccination, either at baseline or TB investigation **(Fig. 2.5)**. Additionally, no differences in total IgG titre were detected between QFT- and QFT+ infants in placebo-treated and vaccination groups **(Fig. 2.5)**.

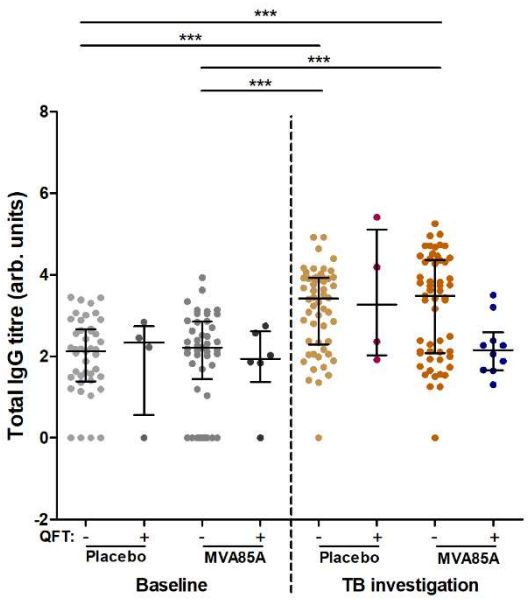

Figure 2.5. Increases in total IgG in QFT negative infants does not associate with MVA85A vaccination status. Relationship between QFT outcome, MVA85A vaccination status and total IgG titres from baseline and TB investigation participant samples. Column sample numbers left-right: 45, 4, 45, 6; 54, 4, 55, 10. Antibody titres are presented as arbitrary values. Comparisons were assessed for significance by the Kruskal-Wallis test, and the frequency of outcomes were compared by the Fisher's exact test.

2.3.4 BCG vaccine-specific antibody responses are not associated with age or QFT outcome

Due to the high TB endemicity in the region, it is recommended that all children be vaccinated with BCG as soon after birth as possible (262). For inclusion in this study, children must have received the BCG vaccine within 7 days of birth. It is known that BCG can induce mycobacteria-specific antibody responses (424-426), so we investigated whether infants enrolled in this study generated an antibody response to the vaccine, measured at TB investigation. BCG-specific antibody responses were detected in a substantial proportion of infant samples: BCG-specific IgG was detected in 93 infants (75.6%) **(Fig. 2.6A)**, IgG1 in 88 (71.54%) **(Fig. 2.6C)**, IgG2 in 32 (26.01%) **(Fig. 2.6E)** and IgG3 in 56 (45.53%) **(Fig. 2.6G)**. To determine whether age-related differences in BCG-specific antibody responses were present, all antibody titres were compared to the infants' age at TB investigation. However, no correlations between age and BCG-specific antibody responses were detected for any of the antibody subtypes tested **(Fig. 2.6B, D, F, H)**.

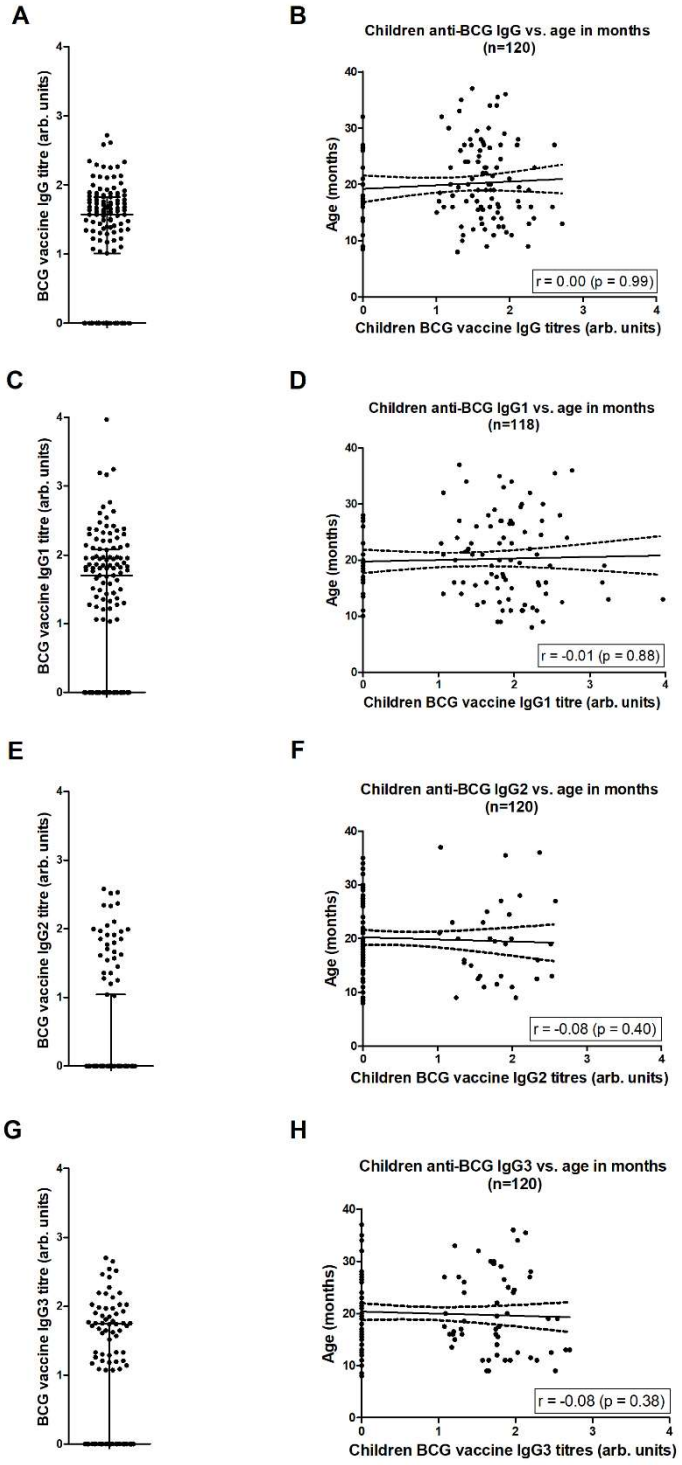

Figure 2.6. Antibody responses to BCG vaccination do not associate with age. BCG-specific antibody responses measured in plasma samples from TB investigation. Anti-BCG IgG (n=123) **(A)**, IgG1 (n=120) **(C)**, IgG2 (n=123) **(E)** and BCG IgG3 (n=123) titres **(G)** as measured by ELISA. Anti-BCG IgG **(B)**, IgG1 **(D)**, IgG2 **(F)** and IgG3 **(H)** as compared to the children's age in months at TB investigation. Three fewer samples reported for BCG IgG1 due to a lack of sample availability. Overlaid **(B, D, F, H)** are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. The strength of the correlations between antibody titres and age was assessed for significance by the Spearman correlation.

Next, BCG vaccine-specific antibody responses were compared to both qualitative and quantitative QFT outcomes at TB investigation. There was no significant association between any of the antibody subtype responses and qualitative QFT outcome **(Fig. 2.7A, C, E, G)**; however, the majority of QFT+ infants did not mount a detectable IgG2 response **(Fig. 2.7E)**. Even though the antibody responses did not associate with reduced risk of *Mtb* infection (as indicated by conversion from QFT- to QFT+), we investigated whether the antibody responses related to changes in IFNγ production. None of the antibody responses exhibited significant correlations with IFNγ production **(Fig. 2.7B, D, F, H),** but there was a trend towards an inverse correlation between BCG-specific IgG3 and quantitative QFT outcome, suggesting that higher antibody responses could be associated with decreased IFNγ production **(Fig. 2.7H)**.

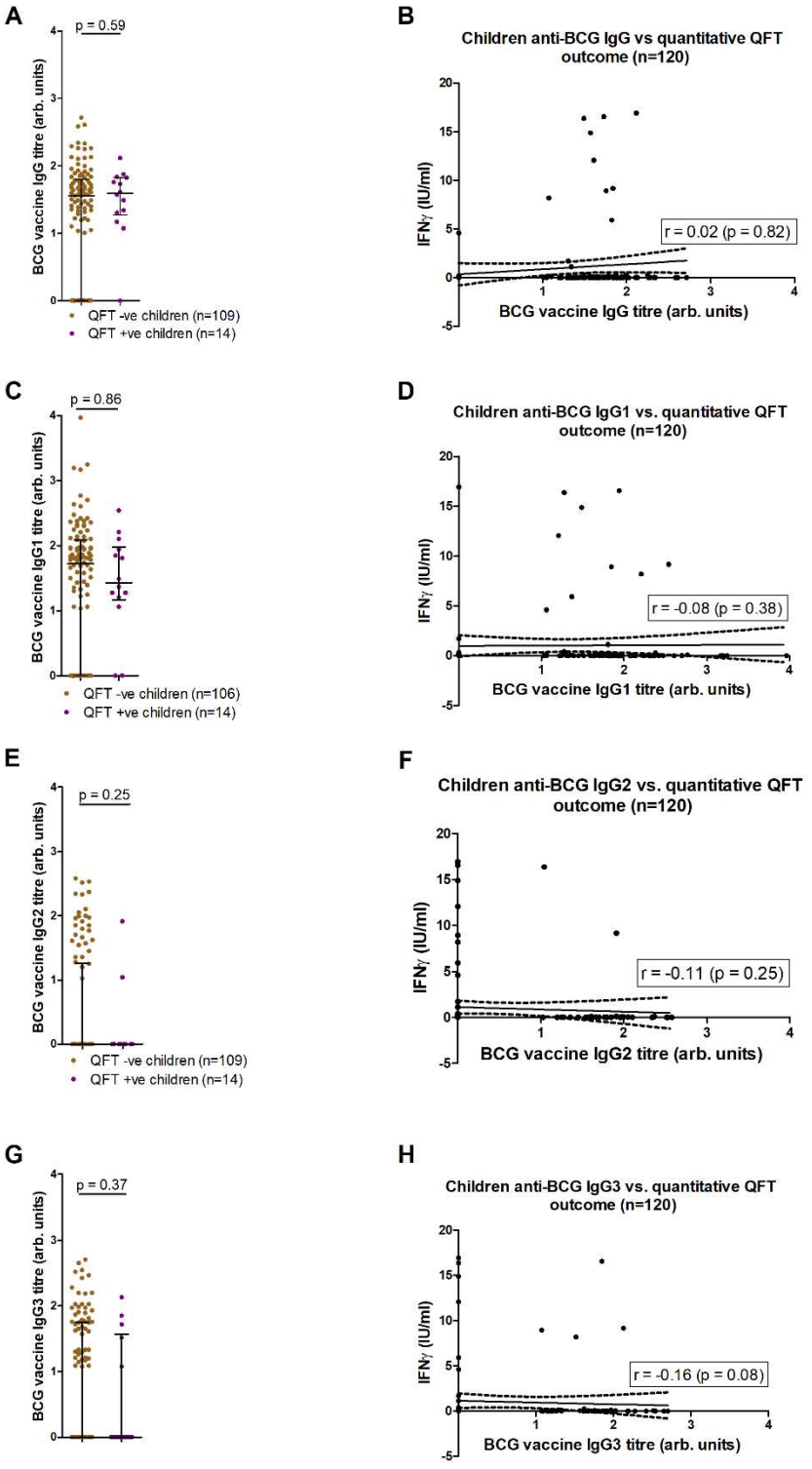

Figure 2.7. Antibody responses to BCG vaccine do not associate with QFT outcome. BCG-specific antibody responses measured in plasma samples from TB investigation and stratified by QFT outcome. Anti-BCG IgG **(A)**, IgG1 **(C)**, IgG2 **(E)** and IgG3 titres as measured by ELISA and stratified by QFT result. Three fewer samples reported for BCG IgG1 due to a lack of sample availability. Anti-BCG IgG **(B)**, IgG1 **(D)**, IgG2 **(F)** and IgG3 **(H)** vs. quantitative QFT outcomes. Overlaid **(B, D, F, H)** are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Comparisons in **(A, C, E, G)** were assessed for significance by the Mann Whitney test; strength of correlations in **(B, D, F, H)** was assessed by the Spearman correlation.

2.3.5 Raised measles vaccine-specific IgG responses associated with a negative QFT outcome but not with age

To investigate whether antibody responses to heterologous antigens from other childhood vaccines related to risk of acquiring an *Mtb* infection, IgG responses to the live-attenuated measles and tetanus toxoid vaccines were measured.

Measles infection is known to induce IgG production which is necessary for long-term immunity, indicating that the induction of robust IgG responses by the live-attenuated measles vaccine is important (195, 413). In this cohort, 116 infants (95.87%) had detectable levels of measles vaccine-specific IgG **(Fig 2.8A)**, but no age-related effects **(Fig 2.8B)** or gender-related effects (data not shown) on the antibody response were observed. As with BCG vaccine-specific responses, potential associations between measles vaccine-specific IgG responses and risk of *Mtb* infection were assessed in plasma samples taken at TB investigation. A trend for decreased measles-specific IgG in infants who acquired *Mtb* infection was detected (p=0.09) **(Fig. 2.8C)**. Although this did not associate with a significant difference in IFNγ produced, a trend for decreased IFNγ levels in infants with higher measles-specific IgG levels was observed **(Fig. 2.8D)**. It is important to note that children younger than 9 months were excluded from these analyses as they would not yet have received their primary measles vaccination.

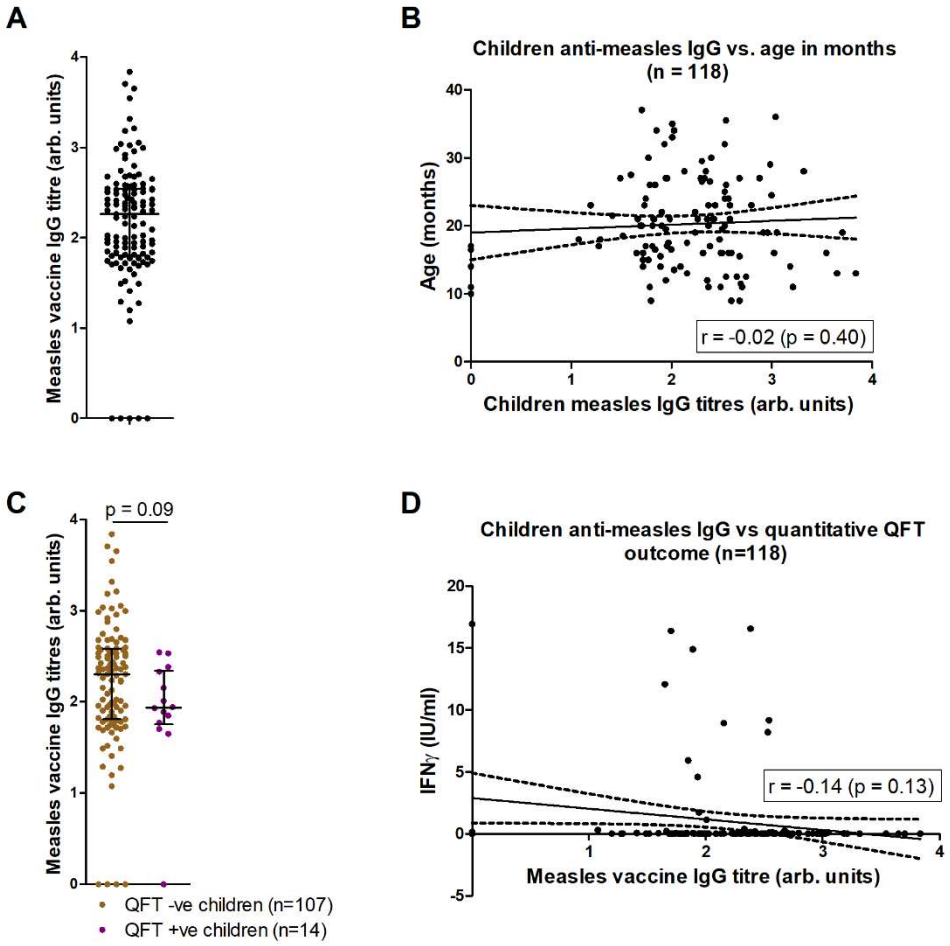

Figure 2.8. Raised measles IgG titres are associated with a QFT negative status. Measles-specific antibody responses measured in plasma samples from TB investigation. Anti-measles IgG (n=121) **(A)** as measured by ELISA. Anti-measles as compared to the children's age in months **(B)**, QFT outcome **(C)** and quantitative QFT outcomes **(D)**. Three fewer samples reported as participants had not yet received the measles vaccine. Overlaid **(B, D)** are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Comparisons in **(A, C)** were assessed for significance by the Mann Whitney test. The strength of the correlations in **(B, D)** was assessed for significance by the Spearman correlation.

2.3.6 Tetanus vaccine-specific IgG responses associated with age but not QFT outcome

Protection against tetanus also requires a robust IgG response, which the tetanus toxoid vaccine is able to induce (413, 427). In this cohort, 101 infants (82.11%) exhibited detectable tetanus vaccine-specific IgG levels **(Fig. 2.9A)** at TB investigation. Numerous booster tetanus vaccines are administered early in life to ensure protective antibody responses are induced (427), so as expected, higher tetanus vaccine-specific IgG titres were significantly correlated with increasing age **(Fig. 2.9B)**. Investigation of tetanus vaccine-

specific responses in light of *Mtb* infection risk revealed no association between IgG responses and either qualitative QFT outcome or IFNγ production **(Fig. 2.9C, D)**.

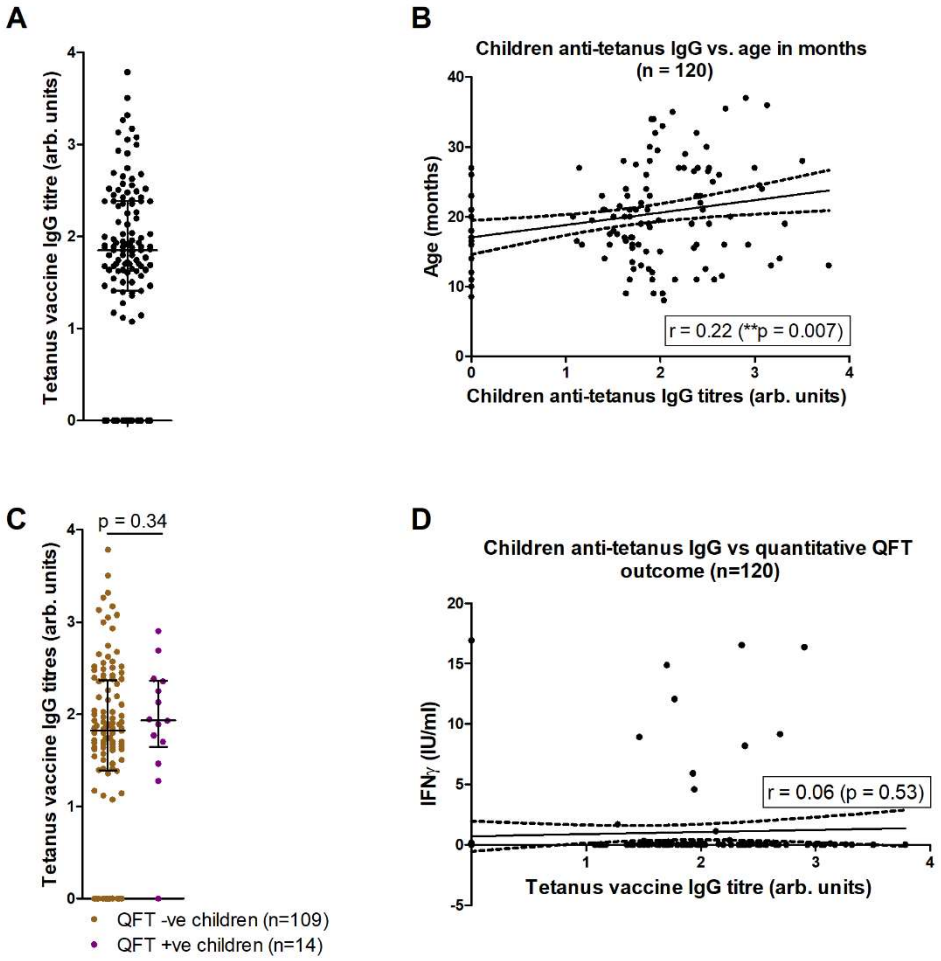

Figure 2.9. Tetanus-specific IgG responses are positively associated with age, but not QFT outcome. Tetanus-specific antibody responses measured in plasma samples from TB investigation. Anti-tetanus IgG (n=123) **(A)** as measured by ELISA. Anti-tetanus as compared to the children's age in months **(B)**, QFT outcome **(C)** and quantitative QFT outcomes **(D)**. Overlaid **(B, D)** are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Comparisons in **(A, C)** were assessed for significance by the Mann Whitney test. The strength of the correlations in **(B, D)** was assessed for significance by the Spearman correlation.

2.3.7 Higher total IgG titres are associated with lower tetanus vaccine-specific IgG responses

To assess whether antibody responses to the BCG, live-attenuated measles and tetanus toxoid vaccines were responsible for the associations observed between total IgG responses and risk of *Mtb* infection at TB investigation, vaccine-specific antibody responses were compared to total IgG titres. These comparisons revealed that neither BCG-specific **(Fig. 2.10A-D)** nor measles-specific **(Fig. 2.10E)** antibody responses correlated with total IgG titres, although a trend for an inverse correlation between BCG-specific IgG1 and total

IgG was observed **(Fig. 2.10B)**. However, there was a significant inverse correlation between tetanus vaccine-specific IgG titres and total IgG titres, suggesting that infants with higher total IgG titres have lower serum tetanus-specific IgG levels **(Fig. 2.10F)**.

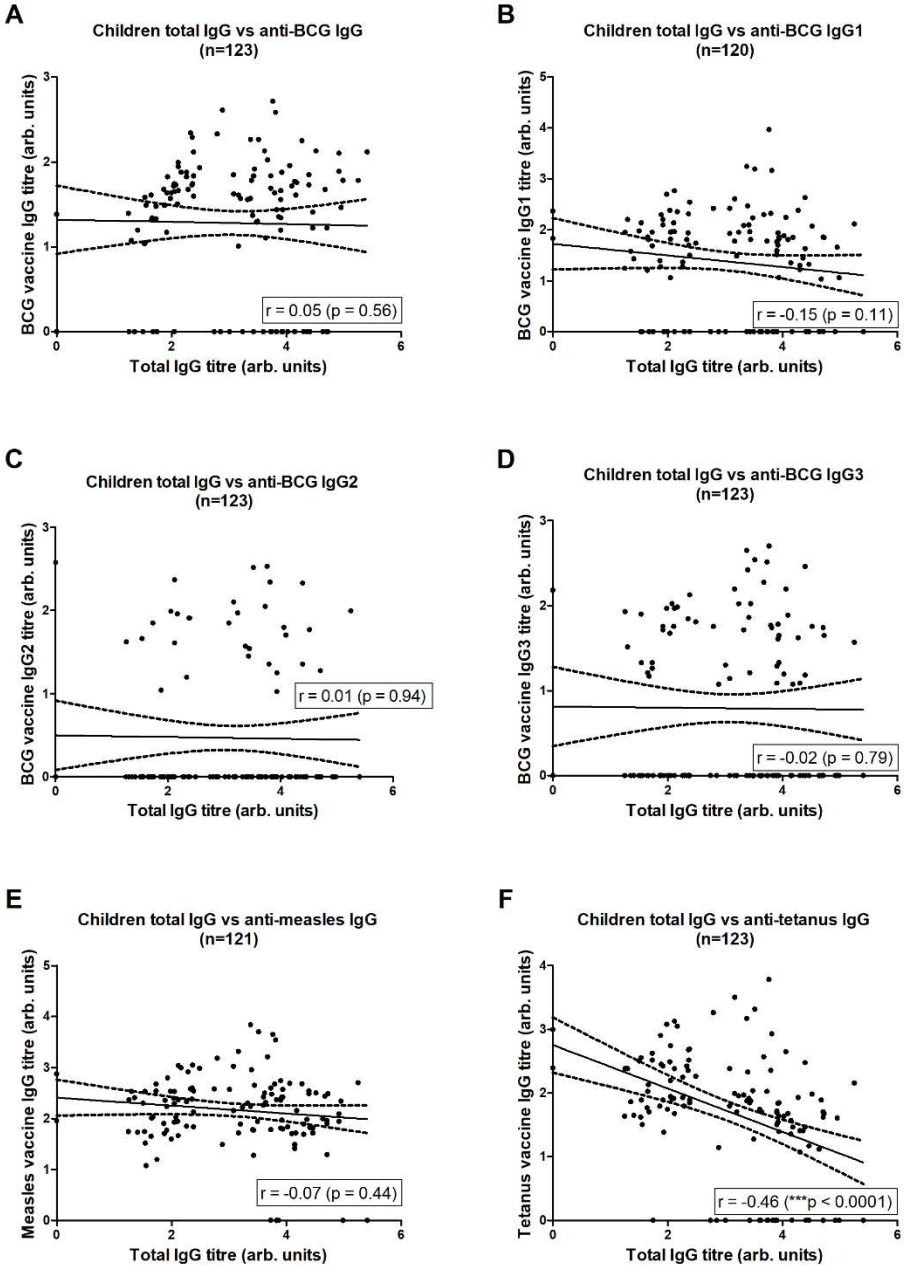

Figure 2.10. Higher total IgG responses are associated with decreased tetanus-specific IgG. Antibody responses measured in plasma samples from TB investigation. Total IgG titres vs. anti-BCG IgG **(A)**, IgG1 **(B)**, IgG2 **(C)**, IgG3 **(D)**, anti-measles IgG **(E)** and anti-tetanus IgG **(F)** titres as measured by ELISA. Three fewer samples reported for BCG IgG1 due to a lack of sample availability, and three fewer reported for measles as participants had not yet received the measles vaccine. Overlaid are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Strength of correlations was assessed by the Spearman correlation.

2.4 Discussion

Taken together, these data show that infants exhibiting a significant increase in plasma total IgG levels from baseline to TB investigation are less likely to be *Mtb*-infected, defined by a QFT+ result. This observed effect was related to a trend for increased BCG vaccine-specific IgG2 responses and measles vaccine-specific IgG in infants who remained *Mtb*-uninfected. These findings suggest that raised total, heterologous and specific IgG responses could be protective against *Mtb* infection. Furthermore, production of protective IgG responses in infants may be due to immune activation by antigens related and unrelated to *Mtb*.

Investigations within a similar cohort have yielded a different outcome. Antigen 85A-specific IgG responses were found to be associated with differences in the prevalence of TB disease but not *Mtb* infection (307). Despite this, it would be inaccurate to compare the two studies directly, due to differences in antigen specificity of the antibody responses measured, assay design and sample size. In accordance with previous research pertaining to the larger cohort from which these infants were recruited, we found no association between MVA85A vaccination and risk of *Mtb* infection (207).

Initial findings indicate that total IgG responses increase significantly from baseline to TB investigation, however at TB investigation there was an inverse correlation between total IgG titres and age. It is important to note that the total IgG titres presented are from two distinct age ranges, namely 4-6 months at baseline and 8-37 months at TB investigation, and that the range of antibody titres values is wide. Additionally, older infants tend to fall within the lower half of the bimodal sample distribution observed at TB investigation, indicating why an inverse correlation between age and antibody titre is possible despite a higher median total IgG titres at TB investigation than at baseline.

Although not significant, a trend for decreased BCG-specific IgG2 titres (as well as a lower proportion of infants producing IgG2) was observed in QFT+ infants, a finding not observed with other BCG-specific IgG responses. BCG vaccination has long been known to induce the production of *Mycobacterium*-specific antibodies (424-426), with IgG1, IgG2 and IgG3 identified as the most prevalent IgG subtypes detected (295, 428). A proportion of infants tested positive for these antibody subtypes, supporting the current literature. However, to our knowledge, no association between a specific IgG subtype and protection against *Mtb*

infection has been identified. Thus, our finding of decreased BCG IgG2 responses in QFT+ infants may warrant further investigation, especially with an increased study population size.

A trend for decreased measles vaccine-specific IgG levels in infants who acquired *Mtb* infection was found; no association was found with tetanus vaccine-specific responses. Measles vaccination has been associated with decreased responsiveness to the tuberculin skin test in individuals with a known positive reaction to purified protein derivative and/or active TB disease (419, 420). However, these studies did not delineate which component of the immune response to measles vaccination could be responsible for these observed effects. Moreover, certain childhood vaccines (such as BCG and measles) have been identified as having a protective effect against all-cause mortality (417, 418). Our finding of higher measles-specific IgG titres in infants who do not acquire an *Mtb* infection provide support for this vaccine being able to elicit heterologous protective effects against unrelated pathogens, such as *Mtb*.

Despite the associations discussed above, it is important to remember that the total IgG response contains antibodies specific for a vast array of antigens not assessed in this study, and the potential role of these other antibody responses in our findings should not be discounted. Another important consideration is the influence of maternal antibody on vaccine responses, especially in younger children. It is widely accepted that the transfer of maternal antibodies during pregnancy and breastfeeding provides neonates with essential protection against pathogens early in life (429). However, the presence of these passively transferred antibodies may also interfere with the infant's immune responses to childhood vaccines (429). The presence of maternal antibody to measles is understood to result in suboptimal induction of protective humoral responses in infants if the measles vaccine is administered too early in life (430, 431). For this reason, administration of the initial measles vaccine is delayed to minimize the impact of maternal antibody (432). As infants in this study were excluded from analyses of measles antibody responses if they had not yet received the primary vaccine, it is reasonable to assume a negligible effect of maternal antibody on these findings. Historically, maternal antibodies to tetanus toxoid have not been thought to interfere with childhood tetanus vaccination (433). However, more recent research has revealed an effect, albeit subtle. Infants exhibiting higher IgG responses to tetanus toxoid at birth also had decreased tetanus toxoid-specific IgG responses following

primary vaccination, suggesting interference by maternally-derived antibody (434). Despite this decrease, the antibody levels induced following vaccination were still considered protective, indicating that the interference by maternal antibody may hold no clinical significance in this instance (434). Although subtle, this effect could provide a plausible explanation for our finding of an inverse association between total IgG titres and tetanus-specific IgG titres. Infants with higher total IgG responses were younger, suggesting that a more pronounced effect of maternal antibodies may be observed in these infants, which in turn could have a detrimental effect on the magnitude of tetanus toxoid-specific IgG responses induced by vaccination. However, the contribution of maternal antibody to the total IgG titres measured would need to be determined for a more definitive conclusion to be drawn.

Another component within the serum antibody fraction that may play a role in heterogenous immune responses is the natural autoantibody component, thought to have a role in the immune effects observed with Intravenous Immunoglobulin (IVIG) treatment (435). IVIG was originally used to treat antibody immune deficiencies, but has also been observed to elicit immunomodulatory effects against autoimmune and other inflammatory disorders (435, 436). The immunoglobulin used is pooled from thousands of donors and mainly consists of IgG, with IgG1 and IgG2 as the most prominent subtypes (435, 436). Antibodies are not only important for protection against pathogens, but also for immune homeostasis, mediated via the action of natural autoantibodies on inhibitory FcRs (435, 437-439). These are a life-long subset of antibodies (IgM, IgA, IgG) generated against certain self-antigens (440-442). As they are suggested to have roles in various anti-inflammatory processes (443), it could be plausible to assume that their function may influence host susceptibility to pathogens by regulating the immune responses required for establishment of infection, such as with *Mtb*. Pertaining to this study, natural autoantibodies could play a role in reducing susceptibility to *Mtb* infection, although it is also important to note that their inhibition of immune components essential for protection could be detrimental (444, 445). However, this is a hypothesis that would require further testing and due to the very limited autoantibody profile present in an individual as compared to the IVIG antibody profile, any potential effects may be negligible.

Another necessary consideration in this study is the impact that small sample size may have had on the findings discussed here. Although the cumulative *Mtb* infection incidence over

the 2 years of follow-up in this study was approximately 11%, the number of infants exhibiting QFT conversion was small, indicating that a larger sample size would be required to investigate these findings more thoroughly. In addition to this, a larger sample size would allow for a group with active TB disease to be included. This study originated as an investigation of the role of standard childhood vaccination in acquisition of *Mtb* infection/active disease, and as such only current vaccine antigens were included. However, the promising protective effects of antibodies elicited against *Mtb*-specific antigens as reported by other researchers (which could be potential vaccine candidates) highlight the importance of future investigations with this or a similar cohort including tests for these *Mtb*-specific antibody responses.

In summary, the data presented here suggest that childhood vaccination with both pathogen-related and unrelated antigens may result in activation of the immature immune system, resulting in reduced risk of *Mtb* infection. The potential role of components within the total IgG response that have not been addressed here should not be discounted, and requires further investigation.

Chapter 3: Investigating the role of helminth exposure on risk of *Mtb* infection

3.1 Introduction

Soil-transmitted helminth (STH) infections are associated with poverty, capable of causing severe morbidity in individuals least likely to have access to appropriate living conditions and adequate healthcare (446). Additionally, children represent a high-risk group for acquiring helminth infections and are often given anti-helminthic treatment at school age in an attempt to alleviate the morbidities associated with these infections, such as nutrient malabsorption, impaired growth and anaemia (446, 447). STHs are known to induce type 2 responses (please refer to section 1.5.2 in Chapter 1), but it remains unclear whether these responses are protective for the host or whether they benefit the parasite (329, 331). Helminth infections in children are associated with increased production of both IgE and IgG4, two type 2 antibodies (397, 404). Whereas IgE is more commonly observed in older children among whom infection levels decrease, IgG4 levels are higher in younger children, where infection levels increase (404). Additionally, these intestinal helminth infections induce the production of type 2 cytokines, although they are suggested to result in decreased cytokine output overall (397).

Due to the often-observed impact and longevity of these infections, it is important to consider all the factors contributing to susceptibility to helminths and co-endemic pathogens, especially the potential modulatory role that these parasites could have on immune responses to unrelated vaccine antigens or co-infections (422, 448-454). The effect of maternal helminth exposure on their offspring's own susceptibility to helminth infection is debated, with some research revealing a detrimental effect (455), and some a beneficial effect (456). Similar complexities are observed when investigating the effect of maternal helminth infection on childhood vaccine responses, again revealing a range of effects from negative to positive (457-461). However, anti-helminthic treatment during pregnancy is suggested to have no lasting impact on the effectiveness of crucial childhood vaccines, implying that maternal helminth infection doesn't have a long-term influence on vaccine responses (462). Although not true of all vaccines investigated (448), the presence of helminth infection at the time of vaccination appears to have a more pronounced effect than maternal helminth exposure, with dampening of vaccine immune response being observed, although not always to a detrimental level (422, 449, 463, 464).

The relationship between helminths and *Mtb* infection/active disease, along with BCG vaccination, is equally complex. Studies of BCG vaccination in both murine models and clinical cohorts reveal seemingly contradictory results. Whilst helminth infection is suggested to dampen protective responses induced by BCG in some instances (465-467), this does not appear to be the case in all conditions (468). Similarly, mycobacterial/helminth co-infection stimulates complex immune responses, dependent on both helminth species and the time at which infection occurs (450, 468-475).

The participants in this study live in a region endemic for *A. lumbricoides* (human roundworm) and *T. trichiura* (human whipworm), and a substantial overlap in prevalence of helminth and *Mtb* infections exists in this region (330, 408). Due to the ability of helminths to affect immune responses to mycobacterial infection, we investigated whether an association existed between helminth exposure (maternal or otherwise) and *Mtb* infection in the infants.

3.2 Materials and methods

The samples used for these analyses are outlined in Chapter 2; additional information and/or modifications are described below.

3.2.1 Determination of helminth infection

In addition to the sample collection described in Chapter 2, informed consent was obtained for the collection of 3 stool samples from the infants during their TB investigation visit. These samples were collected for Kato-Katz stool egg counts (National Health Laboratory Service, Groote Schuur Hospital, Western Cape, South Africa) (476) to determine the prevalence of helminth infection in these infants. Consent was also obtained to collect stool samples from the mothers, and the mothers were asked to complete a questionnaire regarding their and their infant's history of helminth infection and living conditions.

3.2.2 ImmunoCAP® test

The ImmunoCAP® *in vitro* assay was used to detect antigen-specific IgE responses in serum/plasma samples and was used according to the manufacturer's instructions (Thermo Fisher Scientific). Testing services were kindly provided by Professor Michael Levin (Red Cross War Memorial Children's Hospital, Western Cape, South Africa). An *Ascaris suum*-derived antigen that is cross-reactive with *A. lumbricoides* was used to detect antigen-specific IgE (477). We describe responses to this antigen as *Ascaris*-specific.

3.2.3 ELISA

The concentrations at which all antigens and antibodies were used, were optimised specifically for use with this in-house ELISA assay. The procedures outlined below were adapted from similar methods described elsewhere (395, 396, 423, 478).

Helminth antigens

Whole *A. lumbricoides* worms were kindly provided by Prof. Michael Levin (Red Cross War Memorial Children's Hospital), and whole *T. trichiura* worms were kindly provided by Prof. Philip Cooper (St. George's University of London). Worms were treated with a 10X penicillin/streptomycin solution and 1X Amphotericin B (Thermo Fisher Scientific) for 1 hour, following which they were washed in FS 1X PBS. Worm sections (*A. lumbricoides*) or whole worms (*T. trichiura*) were then homogenized in FS 1X PBS before being centrifuged to remove insoluble cellular debris. The soluble fraction was carefully decanted and the protein concentration measured by BCA assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). This soluble fraction was then diluted in FS 1X PBS and adjusted to a stock concentration of 500µg/ml; aliquots of the stock solution were frozen at -80°C until required.

Antigen-specific ELISA

Antigen-specific antibody ELISAs were performed. Coating antigens were used at 5µg/ml or 10µg/ml for antigen-specific IgG or antigen-specific IgG subtypes, respectively. Initial plasma sample dilutions were 1:20 (except for maternal A. lumbricoides-specific IgG which was 1:50), followed by serial 1:5 dilutions of the initial dilutions across a further 7 wells. Primary and detection antibodies were used as described in Chapter 2; all detection antibodies were alkaline phosphatase-linked **(Table 3.1)**.

Table 3.1. ELISA antibodies.

Antibody	Primary/ detection	Dilution	Clone	Company
Mouse α-human IgG (Fc)-AP	Detection	1:1000	JDC-10	Southern Biotech
Mouse α-human IgG4 (pFc')-AP	Detection	1:500	HP6023	Southern Biotech

Nunc-Immuno™ MicroWell™ Maxisorp™ 96-well plates (Thermo Fisher Scientific; Waltham, MA) were coated with antibody or antigen and incubated at 37°C for 3 hours. Plates were washed 3 times with PBS-Tween20 buffer and blocked with 200µl/well 2% milk powder/1X PBS at 4°C overnight. Plates were washed 3 times as above and sample added (50 µl/well); plates were incubated at 4°C overnight. Plates were washed 3 times as above

and secondary antibody added (50 µl/well); plates were incubated at 37°C for 3 hours. Plates were washed 4 times as above and PNP substrate (Sigma-Aldrich; St. Louis, MO) added. Reactions were stopped with 1M NaOH and plates read on a Versamax™ 96-well plate reader (Molecular Devices; Sunnyvale, CA) at wavelength 405nm (492nm reference filter). Arbitrary antibody responses were recorded from sample titration curves as the dilution at which a defined OD value was reached (423). Subsequently, these values were log transformed prior to further analysis to normalise the data, enabling a relative assessment of antibody levels in serum as previously described (423). According to this analysis, antibody levels in certain samples and for certain antibody types fell below the detection limit and are observed at zero on the y-axes. This does not preclude the possibility of specific antibody responses within those samples, but may require detection with a more sensitive assay.

3.2.4 Statistical analysis

Antibody responses in matched sample pairs were compared using the Wilcoxon matched-pairs signed rank test, and the Mann Whitney test was utilized for the analysis of unpaired two-group data. All grouped analyses were investigated by the Kruskal-Wallis test and correlations were investigated using the Spearman correlation test; lines-of-best-fit are overlaid on correlation graphs. Where appropriate, analyses were two-tailed. Data are represented with the median, and significance was accepted at $p \leq 0.05$. GraphPad Prism software (v. 5.03) was used for all statistical analyses and graphs presented.

3.3 Results

3.3.1 Cohort housing, sanitation and helminth infection rates

Of the 123 infants, data regarding housing and sanitation conditions, as well as stool samples, were collected from 113 infants at TB investigation. The majority of infants lived in houses, and had access to clean water (tap) and sanitation (flush toilet) sources **(Table 3.2)**. Stool samples were tested for the presence of helminth eggs, as an indication of current infection. However, none of the samples tested positive for active helminth infection **(Table 3.2)**. As previously mentioned, maternal helminth infections have a complex effect on children's immune responses to helminths, vaccines and unrelated pathogens (455-458, 474). To investigate whether any maternal effects were apparent in this cohort, the prevalence of active helminth infection and helminth-specific antibody responses amongst mothers were determined in samples acquired upon their infant's TB

investigation visit **(Table 3.2)**. Maternal data represents the number of mothers whose serum samples were tested for antibody responses and who also had a stool sample collected. Active helminth infections were tested in several samples, with 1 testing positive for *A. lumbricoides* infection, and 2 for *T. trichiura* infection **(Table 3.2)**.

Table 3.2. Housing, sanitation and helminth infection at TB investigation.

	N= 113 (infants); N=43 (mothers)
Housing type	
Flat	7 (6.19%)
House	77 (68.14%)
Informal settlement	28 (24.78%)
Located on farm (YES)	42 (37.17%)
Source of drinking water	
Communal	1 (0.88%)
Tap water	112 (98.11%)
Sanitation type	
Bucket system	9 (7.96%)
Flush toilet	101 (89.38%)
Pit latrine	3 (2.65%)
Infant prevalent helminth species	
At least one stool sample collected	113 (100%)
A. lumbricoides	0
T. trichiura	0
Other	0
Maternal prevalent helminth species	
At least one stool sample collected	43 (100%)
A. lumbricoides	0
T. trichiura	2 (4.65%)
Other	1 (2.33%)

3.3.2 Infants exhibit an age-related increase in *A. lumbricoides*-specific IgG titres from baseline to time of TB investigation

As mentioned, none of the infants tested positive for active helminth infection **(Table 3.1)**. However, the presence of helminth-specific class-switched antibodies in plasma samples from these infants is indicative of prior helminth exposure. At TB investigation, 2/56 (3.57%) infant samples tested for IgE had detectable levels of *Ascaris*-specific IgE, as determined by ImmunoCAP® **(Fig. 3.1A)**. Analysis of baseline and TB investigation samples for the presence of *A. lumbricoides*-specific IgG revealed that 85/100 (85%) infants at baseline and all infants at TB investigation exhibited detectable antibody levels; furthermore, antibody levels were significantly increased at TB investigation compared to baseline **(Fig. 3.1B)**. Additionally, 61/123 (49.59%) infants at TB investigation had detectable levels of *A. lumbricoides*-specific subtype IgG4 **(Fig 3.1C)**. Analysis of infants' *A. lumbricoides*-specific IgG responses at TB investigation in light of their age at this time-point revealed a significant positive correlation between *A. lumbricoides*-specific IgG and age **(Fig. 3.1D)**. However, no association was found between *A. lumbricoides*-specific IgG4 and age **(Fig. 3.1E)**.

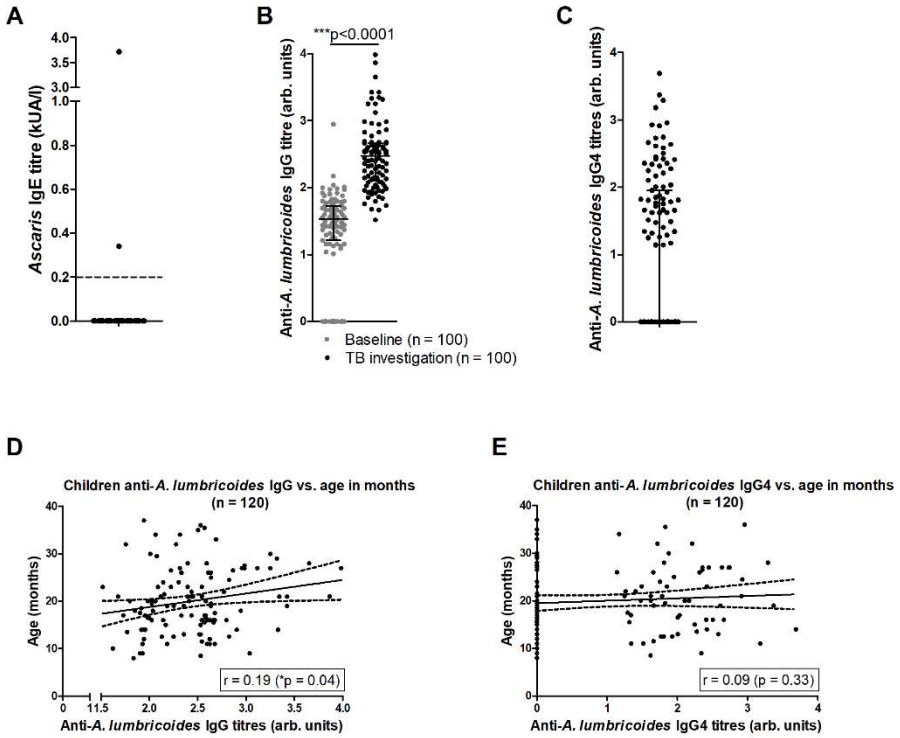

Figure 3.1. Infant *Ascaris*-specific IgG increases from baseline to TB investigation. Anti-*Ascaris* IgE (n=56) **(A)**, IgG **(B)** and IgG4 (n=123) **(C)** titres; values shown in **(A, C)** are from samples taken at TB investigation. Anti-*A. lumbricoides* IgG **(D)** and IgG4 **(E)** titres compared to the participants' age in months at TB investigation. Overlaid in **(D, E)** are the lines-of-best-fit and the 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Comparison in **(B)** was assessed for significance by the Mann Whitney test. The strength of the correlations in **(D, E)** was assessed for significance by the Spearman correlation.

3.3.3 *A. lumbricoides*-specific antibody responses are not associated with QFT outcome

Due to the potential of helminth exposure to alter immune responses to *Mtb* infection and active disease (469, 475, 479), any potential association of infant *A. lumbricoides* exposure with risk of *Mtb* infection was investigated. There were no significant differences when either baseline or TB investigation *A. lumbricoides*-specific IgG responses were stratified by QFT outcome **(Fig. 3.2A)**. Further longitudinal analysis of individual *A. lumbricoides*-specific IgG titres in QFT- and QFT+ infants revealed significant increases from baseline to TB investigation in both groups, indicating an increase in *A. lumbricoides*-specific IgG levels irrespective of acquisition of an *Mtb* infection **(Fig.3.2B)**. Similarly, *A. lumbricoides*-specific IgG4 responses did not associate with QFT conversion **(Fig. 3.2C)**. It has been observed that helminth infection can influence the magnitude of the QFT IFNγ response negatively, with an indeterminate result becoming more likely (480, 481). However, in this cohort, no

correlation was found between *A. lumbricoides*-specific antibody responses and IFNγ measured by QFT assay **(Fig. 3.2D, E)**.

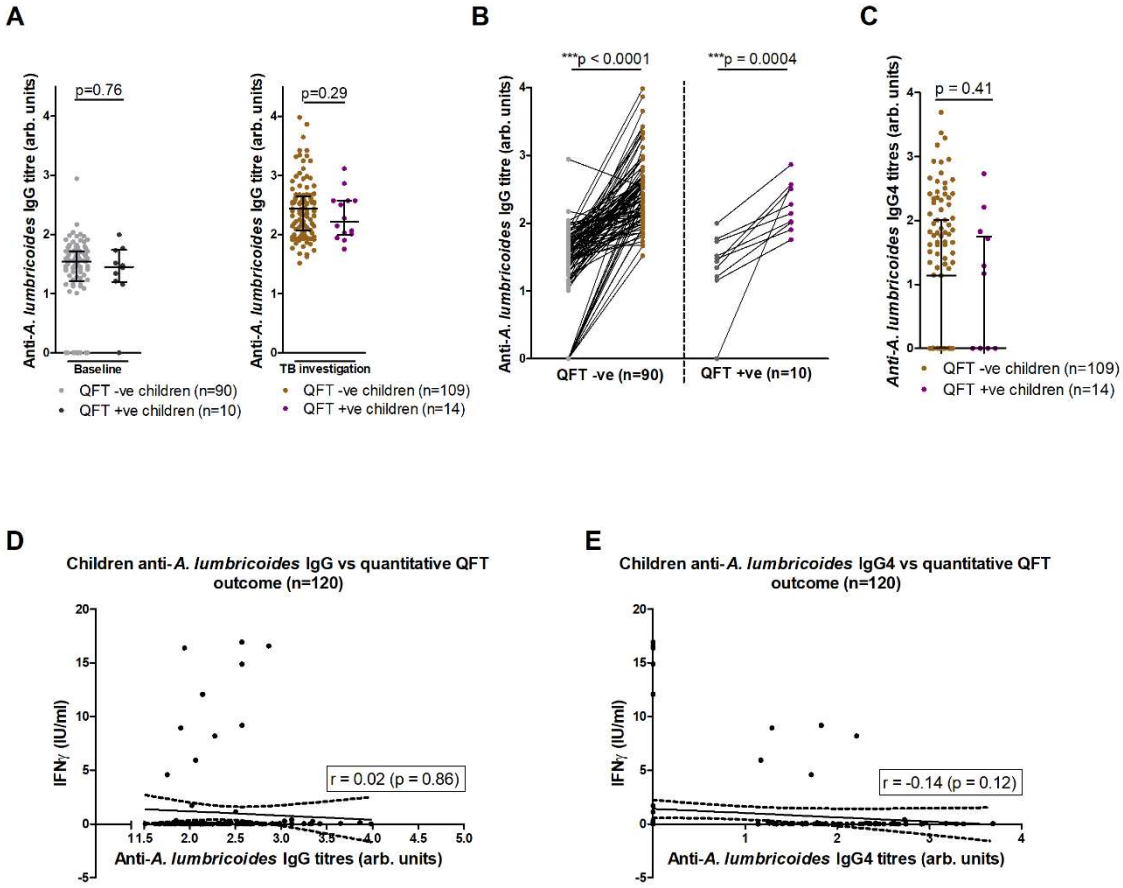

Figure 3.2. *A. lumbricoides*-specific IgG and IgG4 do not associate with QFT outcome at TB investigation. Anti-*A. lumbricoides* IgG titres stratified by QFT outcome **(A)**; **(B)** is a before/after comparison of TB investigation anti-*A. lumbricoides* IgG titres as subdivided in **(A)**. Anti-*A. lumbricoides* IgG4 titres stratified by QFT outcome **(C)**. Anti-*A. lumbricoides* IgG **(D)** and IgG4 **(E)** titres vs. quantitative QFT outcomes. Overlaid in **(D, E)** are the lines-of-best-fit and the 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Comparison in **(A)** was assessed for significance by the Kruskal-Wallis test **(B)**, and comparisons in **(B)** by the Mann Whitney test. The strength of the correlations in **(D, E)** was assessed for significance by the Spearman correlation.

3.3.4 *T. trichiura*-specific antibody responses are not associated with QFT outcome

Further analysis of plasma samples revealed a class-switched antibody response to the human whipworm, *T. trichiura*. At TB investigation, 97/123 (78.86%) infants had detectable *T. trichiura*-specific IgG responses **(Fig. 3.3A)**, and 59/123 (47.97%) infants had detectable IgG4 responses **(Fig. 3.3C)**. The *T. trichiura*-specific antibody responses were compared to the infants' age at the time of sample collection, but no correlation was found between age and either IgG or IgG4 antibody responses **(Fig. 3.3B, D)**.

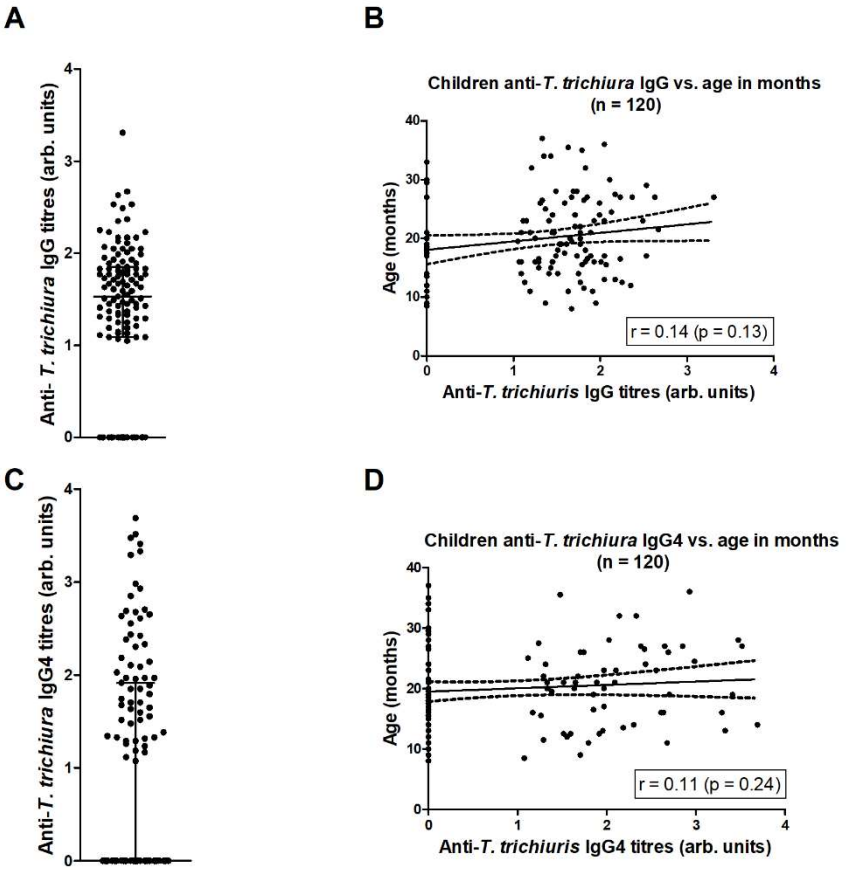

Figure 3.3. *T. trichiura*-specific IgG and IgG4 do not associate with age. *T. trichiura*-specific antibody responses measured in plasma samples from TB investigation. Anti-*T. trichiura* IgG (n=123) **(A)** and IgG4 (n=123) **(C)** titres. Anti-*T. trichiura* IgG **(B)** and IgG4 **(D)** titres compared to the participants' age in months at TB investigation. Overlaid in **(B, D)** are the lines-of-best-fit and the 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. The strength of the correlations in **(B, D)** was assessed for significance by the Spearman correlation.

As with *A. lumbricoides*-specific responses, the *T. trichiura* responses at TB investigation were stratified by QFT outcome. There was no association between either IgG or IgG4 antibody responses and QFT outcome, with similar median antibody responses in QFT- and QFT+ infants **(Fig. 3.4A, B)**. There was also no association between either *T. trichiura*-specific IgG or IgG4 responses and IFNγ production as measured by the QFT assay **(Fig. 3.4C, D)**.

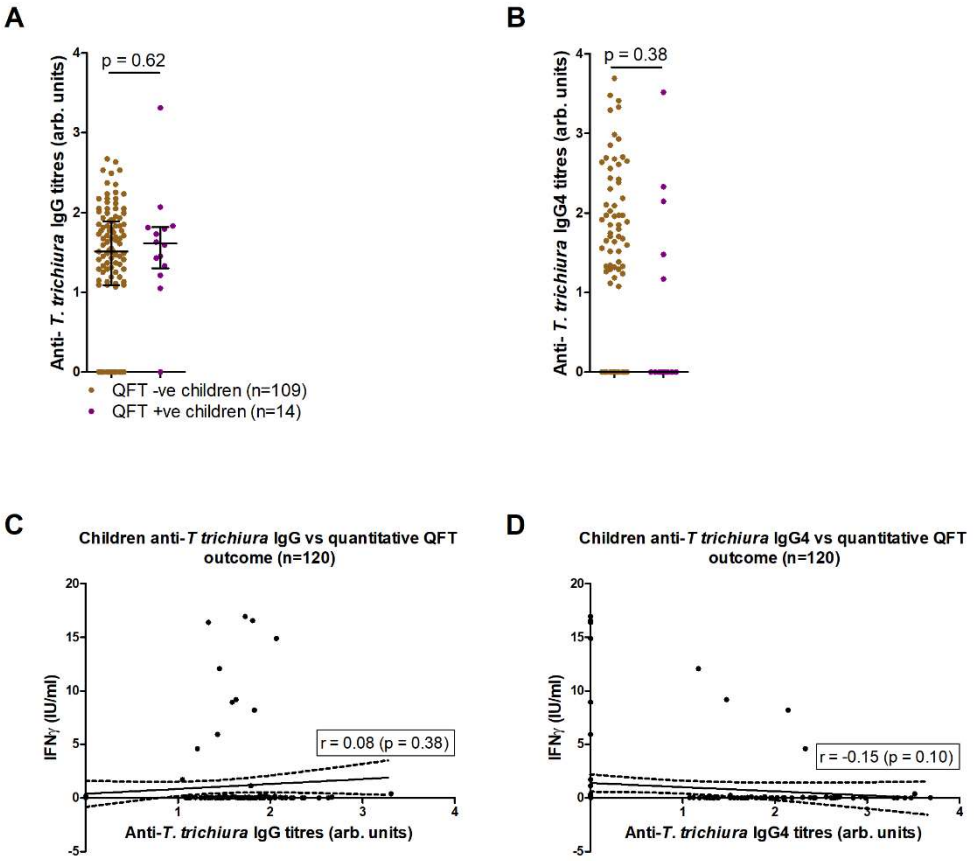

Figure 3.4. *T. trichiura*-specific IgG and IgG4 do not associate with QFT outcome. *T. trichiura*-specific antibody responses measured in plasma samples from TB investigation. Anti-*T. trichiura* IgG (A) and IgG4 (B) titres stratified by QFT outcome. Anti-*T. trichiura* IgG (C) and IgG4 (D) titres vs. quantitative QFT outcomes. Overlaid in (C, D) are the lines-of-best-fit and the 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Comparisons in (A, B) were assessed for significance by the Mann Whitney test. The strength of the correlations in (C, D) was assessed for significance by the Spearman correlation.

3.3.5 Maternal and infant helminth-specific antibody responses are variable

The presence of *A. lumbricoides*-specific IgE, IgG and IgG4, as well as *T. trichiura*-specific IgG4 antibodies in maternal serum samples taken at the time of their infants' TB investigation visit were also measured. *A. lumbricoides*-specific IgE responses were detected in 13/44 (29.55%) maternal samples tested, as determined by ImmunoCAP® **(Fig. 3.5A)**. A higher number of maternal samples could be tested for the presence of helminth-specific IgG and IgG4 antibodies, thus more descriptive pairwise comparisons with matched infant samples were subsequently performed. Following analysis of subsets of 76 mother-infant pairs (baseline infant samples) and 89 mother-infant pairs (TB investigation infant samples), it was observed that maternal *A. lumbricoides*-specific IgG responses were significantly higher than those observed in infants **(Fig. 3.5B, C)**. Analysis of *A. lumbricoides*-specific IgG4 responses in a subset of 49 mother-infant pairs (TB investigation infant

samples) revealed significantly higher median responses in infants, with a greater proportion of infants producing IgG4 **(Fig 3.5C)**. In the subset of 34 mother-infant pairs investigated for *T. trichiura*-specific IgG4 responses (TB investigation infant samples), infants again exhibited significantly higher median IgG4 responses than their matched mothers, with a greater proportion of infants producing detectable IgG4 titres **(Fig. 3.5E)**.

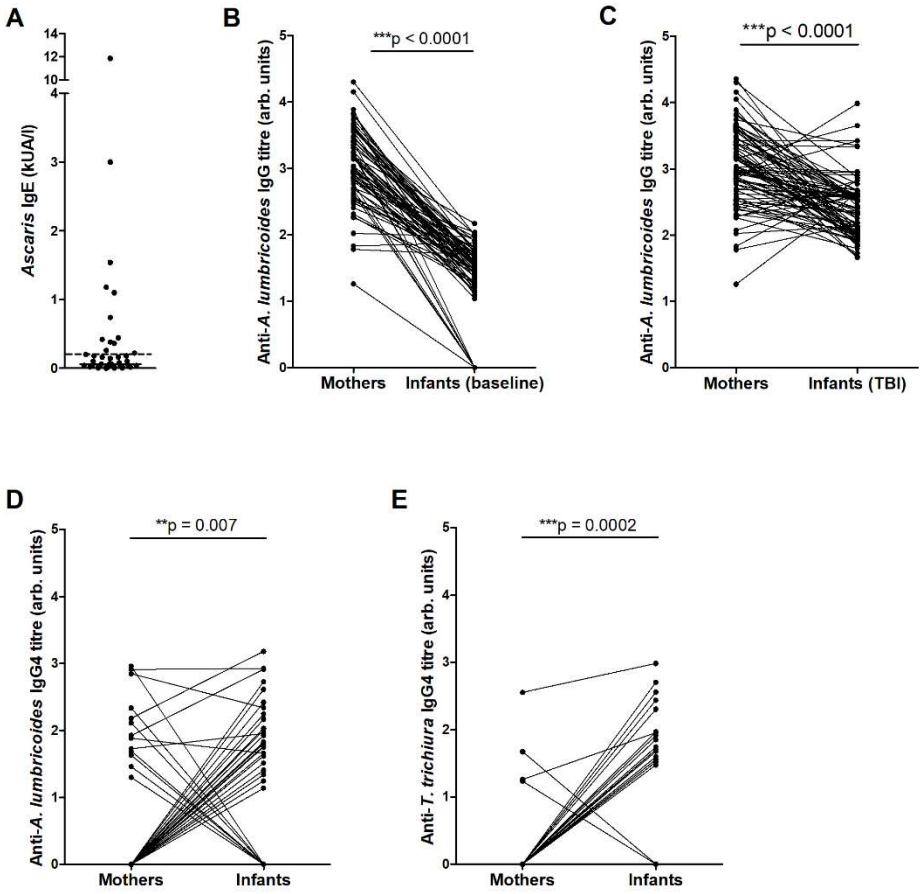

Figure 3.5. Variable associations exhibited between maternal and infant helminth-specific antibody responses. *A. lumbricoides* and *T. trichiura*-specific IgG and IgG4 in maternal serum samples taken at infants' TB investigation visit versus infant helminth-specific IgG and IgG4 in plasma samples from baseline and TB investigation. Maternal anti-*Ascaris* IgE (n=44) **(A)**; dashed line represents assay detection limit for positive responses. Maternal *A. lumbricoides*-specific IgG vs. infant *A. lumbricoides*-specific IgG at baseline (n=76 pairs) (maternal sample dilutions started at 1:50 and infants' at 1:20) **(B)** and vs. infant IgG at TB investigation (n=89 pairs) **(C)**. Maternal *A. lumbricoides*-specific IgG4 vs. infant *A. lumbricoides*-specific IgG4 at TB investigation (n=49 pairs) **(D)**. Maternal *T. trichiura*-specific IgG4 vs. infant *T. trichiura*-specific IgG4 at TB investigation (n=34 pairs) **(E)**. Antibody titres are presented as arbitrary values. Comparisons in **(B-E)** were assessed for significance by the Mann Whitney test.

It is still debated to what extent maternal helminth infection influences infant cytokine responses to specific antigens (458, 460, 462, 474). To investigate whether maternal helminth exposure (as opposed to active infection) plays a role, infant *Mycobacterium*

antigen-specific IFNγ production was analysed according to maternal helminth-specific antibody levels. However, there was no association between *A. lumbricoides*-specific IgG or IgG4 responses and infant T cell IFNγ production as measured by QFT **(Fig. 3.6)**.

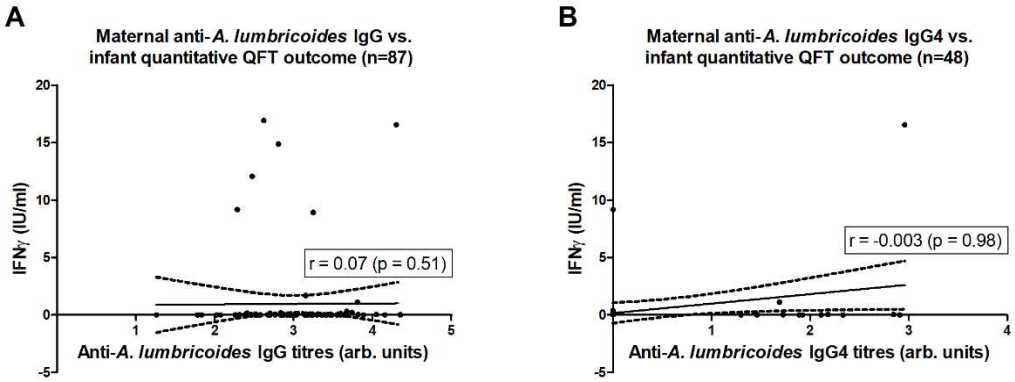

Figure 3.6. Maternal *A. lumbricoides*-specific IgG and IgG4 do not associate with infants' quantitative QFT values. *A. lumbricoides*-specific IgG and IgG4 measured in maternal serum samples taken at infants' TB investigation visit versus quantitative outcomes of infant TB investigation visit QFT test. Anti-*A. lumbricoides* IgG **(A)** and IgG4 **(B)** titres vs. quantitative QFT outcomes. Overlaid are the lines-of-best-fit and the 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. The strength of the correlations was assessed for significance by the Spearman correlation.

3.3.6 Infant, but not maternal, helminth-specific antibody responses are associated with childhood vaccine antibody responses

As maternal helminth exposure and concurrent helminth infection are suggested to have different effects on vaccination responses (458, 459, 463), total and vaccine-specific antibody titres in the infants were investigated in light of their own helminth exposure, as well as maternal helminth exposure. No associations were found between helminth-specific antibody responses and total IgG responses **(Fig. 3.7A, B)**, but significant positive correlations were found between *A. lumbricoides*-specific **(Fig. 3.7C-E)** and *T. trichiura*-specific **(data not shown; *A. lumbricoides*-specific responses are representative of both helminths)** IgG responses and IgG responses to the BCG, measles and tetanus vaccines. No association was found between any of the BCG-specific antibody subtypes tested and helminth-specific IgG except for BCG-specific IgG1 which was associated with *T. trichiura*-specific IgG **(Fig. 3.7F)**.

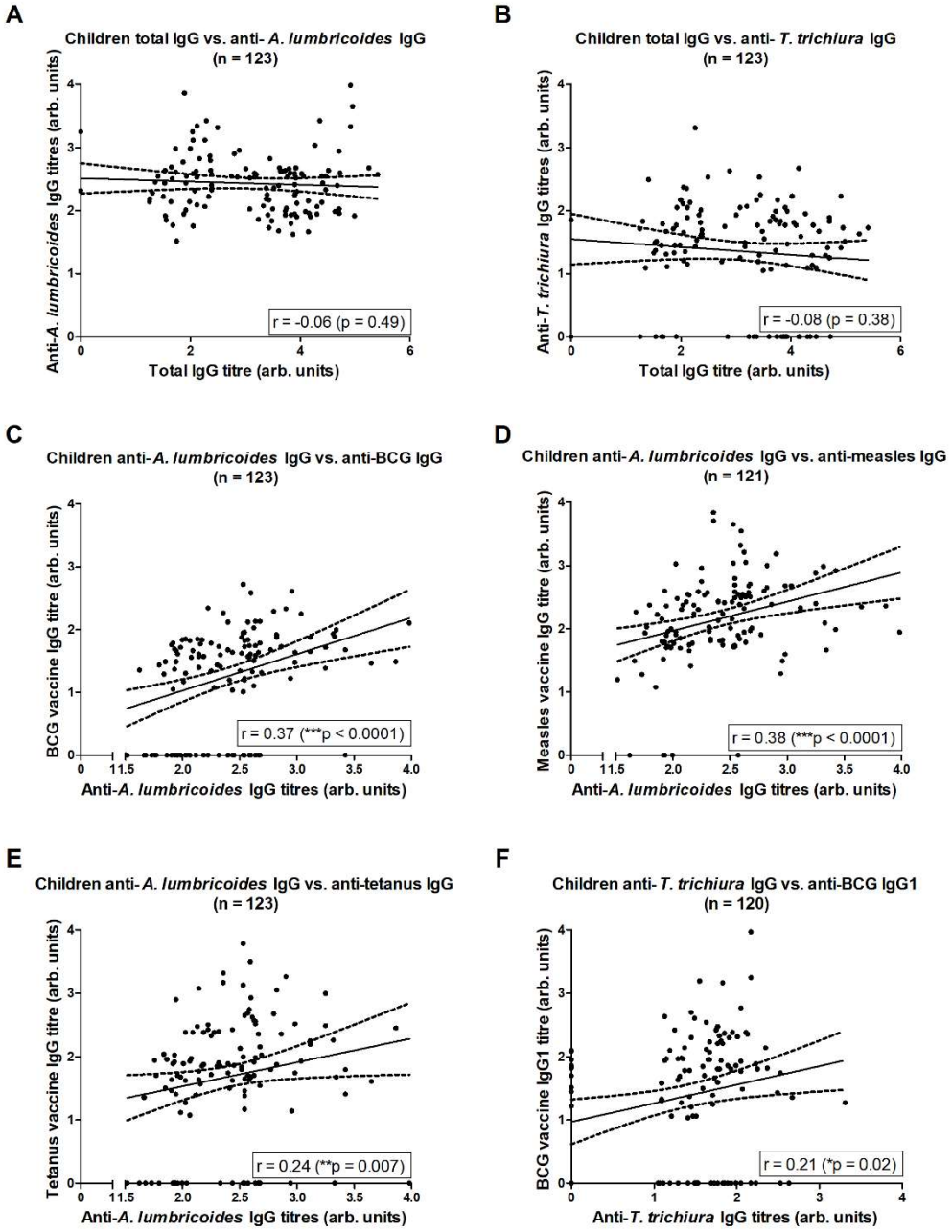

Figure 3.7. Infant helminth-specific IgG is positively associated with antibody responses to childhood vaccines. Helminth and vaccine-specific antibody responses measured in plasma samples from TB investigation. *A. lumbricoides*-specific IgG **(A)** and *T. trichiura*-specific IgG **(B)** titres vs. total IgG titres. *A. lumbricoides*-specific IgG vs. anti-BCG IgG **(C)**, anti-measles IgG **(D)** and anti-tetanus IgG **(E)**; *T. trichiura*-specific IgG vs. anti-BCG IgG1 **(F)** titres as measured by ELISA. Three fewer samples reported for measles as participants had not yet received the measles vaccine, and three fewer reported for BCG IgG1 due to a lack of sample availability. Overlaid are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Strength of correlations was assessed by the Spearman correlation.

Vaccine-specific antibody titres were analysed in light of helminth-specific subtype responses. As with the IgG responses **(Fig. 3.7A, B)**, helminth-specific IgG4 responses did not associate with total IgG titres (data not shown). However, both *A. lumbricoides*-specific and *T. trichiura*-specific **(data not shown; *A. lumbricoides*-specific responses are representative of both helminths)** IgG4 responses were significantly correlated with BCG-specific IgG, IgG1, IgG2 and IgG3 titres **(Fig. 3.8A-D)**, as well as with measles- and tetanus-specific IgG responses **(Fig. 3.8E, F)**.

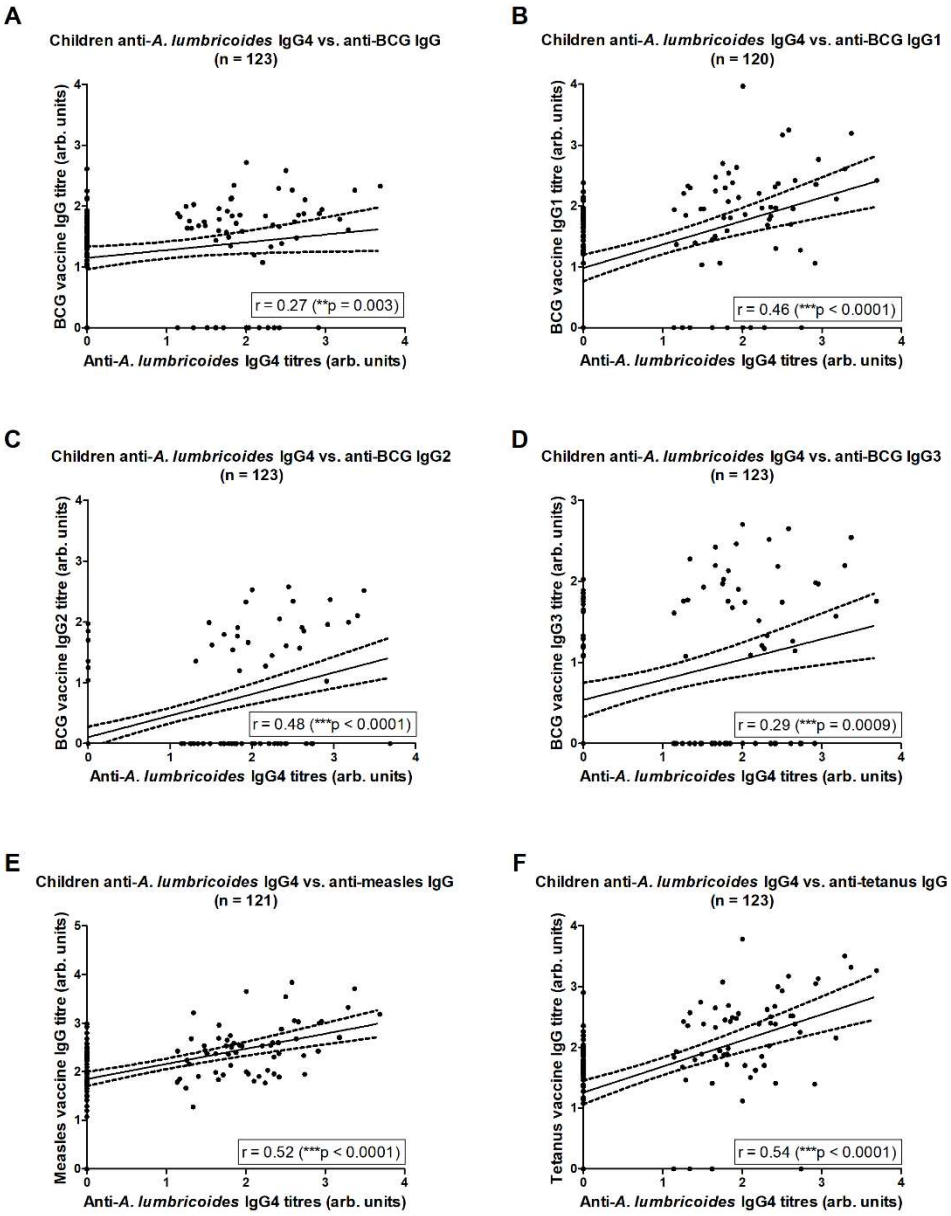

Figure 3.8. *A. lumbricoides*-specific IgG4 is positively associated with childhood vaccine antibody responses. Helminth and vaccine-specific antibody responses measured in plasma samples from TB investigation. *A. lumbricoides*-specific IgG4 titres vs. anti-BCG IgG **(A)**, IgG1 **(B)**, IgG2 **(C)**, IgG3 **(D)**, anti-measles IgG **(E)** and anti-tetanus IgG **(F)** titres as measured by ELISA. Three fewer samples reported for BCG IgG1 due to a lack of sample

availability, and three fewer reported for measles as participants had not yet received the measles vaccine. Overlaid are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Strength of correlations was assessed by the Spearman correlation.

To determine whether maternal-infant antibody transfer of helminth-specific antibodies could be responsible for these positive associations, maternal helminth-specific antibodies were compared to infant vaccine antibody responses. Unlike the infant helminth-specific responses, maternal *A. lumbricoides*- and *T. trichiura*-specific responses did not associate with the infants' antibody responses to the BCG, measles or tetanus vaccines **(Fig. 3.9; *A. lumbricoides*-specific IgG responses are representative of *A. lumbricoides*- and *T. trichiura*-specific IgG4 responses)**.

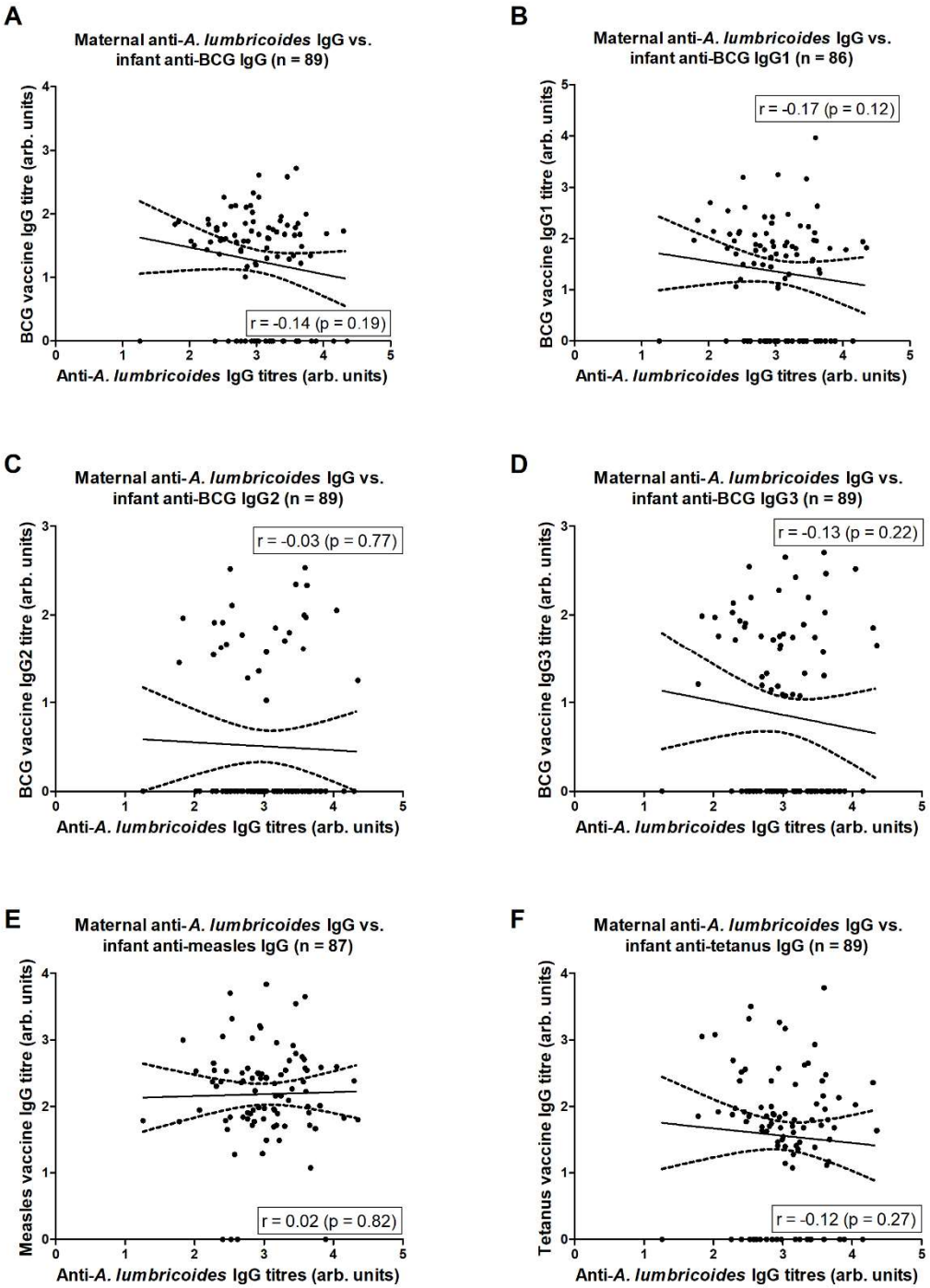

Figure 3.9. Maternal *A. lumbricoides*-specific IgG responses are not associated with infant childhood vaccine responses. *A. lumbricoides*-specific IgG in maternal serum samples taken at infants' TB investigation visit versus infant vaccine-specific antibodies in plasma samples from TB investigation. Maternal *A. lumbricoides*-specific IgG vs. anti-BCG IgG **(A)**, IgG1 **(B)**, IgG2 **(C)**, and IgG3 **(D)** titres; maternal A. lumbricoides-specific IgG vs. anti-measles IgG **(E)** and anti-tetanus IgG **(F)** titres. Three fewer samples reported for BCG IgG1 due to a lack of sample availability, and three fewer reported for measles as participants had not yet received the vaccine. Overlaid are the lines-of-best-fit and 95% confidence bands (dashed lines). Antibody titres are presented as arbitrary values. Strength of correlations was assessed by the Spearman correlation.

3.4 Discussion

Findings from this study reveal that despite the fact that these mothers and infants live in a region endemic for helminth infection (330), no current *A. lumbricoides* or *T. trichiura* infections were detected in the infants, and the rate of infection in mothers was low. This could be due to the availability of appropriate sanitation and water sources **(Table 3.2)** (482, 483), as well as mass deworming campaigns in the community (484).

Despite low levels of active infection in the participants tested, helminth exposure is observed in this population, as indicated by the presence of antigen-specific class-switched antibodies. Associations between infant helminth-specific antibody responses and age were dependent on both helminth species and antibody subtype, whereas associations with vaccine responses did not show the same limitations. However, there was no association between helminth-specific antibody responses and either risk of *Mtb* infection or total IgG responses, nor between maternal helminth-specific and infant vaccine-specific responses. Taken together, these observations suggest that, in this setting, helminth exposure does not influence the risk of acquiring an *Mtb* infection, nor that the humoral response to the helminth antigens tested underlies the association observed between total IgG responses and risk of *Mtb* infection, but we substantiate previous findings that soil-transmitted helminths may influence infant vaccination.

A higher number of mothers than infants exhibited *Ascaris*-specific IgE production, with mothers also exhibiting greater levels of *A. lumbricoides*-specific IgG, a finding which may be accounted for by the presence of *A. lumbricoides*-specific IgG1 (395); however, this would need to be tested to confirm whether this is true of our cohort. Conversely, more infants produced *A. lumbricoides*- and *T. trichiura*-specific IgG4, with the IgG4 titres measured being significantly higher than those observed in maternal samples. These findings are in agreement with prior reports that young children exhibit raised helminth-specific IgG4, and older children and adults raised IgE (404, 485), but contradict a report showing no relationship between maternal and neonatal *A. lumbricoides*-specific IgG4 levels, with most neonatal samples testing positive for *A. lumbricoides*-specific IgG4 seemingly unrelated to maternal infection status (486). Further investigation would be required to determine whether these observations represent a causal relationship. The increased levels of helminth-specific antibodies in the infants could also be explained by passive antibody transfer (456). However, due to the wide age range (8-36 months) of

infants at the time of testing for helminth-specific antibodies, it is difficult to assess whether these antibodies were present due to passive transfer or helminth exposure alone. Additionally, the lack of current infection limits our ability to investigate whether these antibodies, passively transferred or not, could provide protection against helminth infection.

The current understanding of the effect of helminth infection/exposure on *Mtb* infection remains unclear. In rodent models, the effects of helminth infection on mycobacterial infection has been reported as neutral (468, 470, 471, 473), negative (469, 472) or positive (450), with similarly complex findings reported in clinical studies (472, 474, 475). However, it is important to consider the effect that helminth species, time of helminth infection (acute versus chronic) and stage of mycobacterial infection (latent vs. active disease) could have on the outcomes reported in these studies, as immune responses are contingent on these factors. For example, helminth infection may not influence immune responses to or acquisition of latent *Mtb* infection in certain populations (487, 488), whereas they could play a role in active TB disease in certain instances (472). In this study, no association was found between either *A. lumbricoides*- or *T. trichiura*-specific antibody responses and risk of *Mtb* infection, with similar median antibody titres in QFT- and QFT+ infants, which is in agreement with the literature reporting a neutral effect of helminth infection/exposure on mycobacterial infection. This finding also suggests that the humoral response to the helminth antigens tested does not account for the differences observed in risk of acquiring an *Mtb* infection when compared to total IgG responses **(shown in Chapter 2)**, a suggestion supported by a lack of association between helminth-specific antibody responses and total IgG titres.

Previous studies report an association between helminth infection and the outcome of a QFT test, with more indeterminate results being observed when helminth infection is present (480, 481). However, neither *A. lumbricoides*- nor *T. trichiura*-specific humoral responses from this study, in mothers or infants, were associated with IFNγ production as measured by QFT. Despite the differing results, it is important to consider that the effects reported in the literature are helminth species-dependent, and may only be observed in the presence of active infection and not helminth exposure.

It has been postulated that the immunomodulatory effects induced by helminths or their antigenic products can result in an impaired response to vaccination (489). Several

published reports are in agreement regarding this, with protective responses to tetanus (422, 463), cholera (449, 464) and BCG vaccines (465-467) suggested to be downregulated by helminth infection. However, a detrimental outcome is not always observed (468, 490). A comparison of infant humoral responses to helminths and childhood vaccines in this study revealed positive associations between antibody responses to both helminths and all vaccines tested, including BCG, measles and tetanus, but only tetanus-specific IgG was also correlated with total IgG **(refer to Chapter 2)**. Despite the importance of a robust humoral response to most childhood vaccines (413), the interaction of helminth infection with antibody responses to vaccine antigens is not always addressed. Where vaccine humoral responses are investigated, there is no consensus on their relation to helminth infection. Vaccine antibody responses are either suggested to be prone to immunomodulation by helminths (422, 464), or not affected at all (490). Despite the positive correlations between helminth and vaccine humoral responses described above, neither of them underlie the association between total IgG responses and reduced risk of *Mtb* infection addressed previously (refer to Chapter 2). The potential benefits of a mycobacterial-specific antibody response to BCG have only recently come to light (285), are not often addressed in the literature (465, 468, 490) and are not yet well understood. As such, we cannot say definitively whether an increased BCG-specific response that is associated with the humoral response to helminths, equates to enhanced protection against *Mtb*. Finally, the observation that maternal helminth-specific antibody responses do not associate with the infants' IgG responses to childhood vaccination support findings published previously (457, 459, 462).

Taken together, these data show that despite low overall rates of helminth infection, the presence of class-switched helminth-specific antibodies in mothers and infants indicates prior helminth exposure. However, this exposure does not relate to the risk of *Mtb* infection acquisition or IFNγ production by *Mycobacterium*-specific T cells, and its effect on vaccine responses is suggested to be minimal. These findings suggest that the immunomodulatory effects often shown to be induced by helminths are associated with active infection, whereas helminth exposure (maternal or otherwise) may have a more subtle effect on the host immune system.

Chapter 4: An *in vivo* model investigating the influence of helminth exposure on BCG vaccination and infection

4.1 Introduction

Our clinical investigation touched on the role of maternal influences on infant immunity (please refer to Chapter 3). An original aim of the clinical component of this research was to investigate the role of maternal helminth infection on infant risk of acquiring an *Mtb* infection; however, with the clinical and sample data available, we were limited in what we could pursue. Paired with this limitation is the scarcity of clinical data addressing the impact of maternal helminth infection on infant *Mtb* infection outcomes. These cross-sectional study outcomes are restricted to measuring cytokine and/or antibody responses to relevant antigens, limiting inferences that can be drawn regarding how *Mtb* infection risk would be affected by alterations of these responses (458, 460, 462, 474). A murine model of maternal helminth infection and infant mycobacterial infection represents an elegant way of overcoming these limitations, and thus was used to address the study questions.

Immune responses induced by *Mtb* are complex; *in vivo* models of mycobacterial infection and disease provide mechanistic insight that allows us to understand the significance of observations made in clinical studies. Commonly used laboratory mouse strains (e.g. BALB/c, C57BL/6) are resistant to *Mtb* infection, limiting bacterial growth and tissue damage, a phenotype somewhat reflective of the response in humans exposed to *Mtb* (491, 492). Moreover, robust Th1 responses required for protection against *Mtb* infection are observed in both humans and mice (493). Thus, considering the benefits of a murine model and the similarities in immune responses to *Mtb* in mice and humans, murine TB models are appropriate models for clinical TB (493), albeit with acknowledged limitations (494, 495). BCG vaccination also elicits protective responses in mice to subsequent *Mtb* infection, even with clinical *Mtb* strains (496, 497). As such, murine models have often been used to understand immune responses induced by BCG vaccination. Additionally, murine BCG infection models are also useful for understanding primary *Mtb* infection in humans (498), while circumventing the practical challenges involved with clinical *Mtb* animal models. Successful BCG infection can be induced in genetically resistant mouse strains (499), it is non-pathogenic (500), and can be controlled by the host (unlike *Mtb* infection) (501). Its uptake to the lungs and spleen mimic that of virulent mycobacterial infection, with

protection characterized by classical Th1 responses (as outlined in section 1.4.1 of Chapter 1) (498, 501).

Nippostrongylus brasiliensis (Nb), a rodent helminth, is commonly used as a model of human helminth infections, such as hookworms (502, 503). This parasite induces potent Type 2 immune responses upon infection, from mucous and cytokine production (IL-4, IL-5, IL-13), to eosinophilia (described in more detail in section 1.5.2 of Chapter 1) (502, 504). Some of these induced components are required for parasite clearance (364), whilst others contribute to wound healing (505). Importantly, although the patent infection is located in the intestine, establishment of patency requires larval migration through the lung (503, 506). Their movement through the lung tissue causes physical damage (breakdown of alveoli), resulting in decreased lung function (507); this is also an important feature of certain human helminth infections (please refer to section 1.5.1 of Chapter 1). Additionally, these helminths and their excretory/secretory (NES) products induce type 2 responses in the lung, stimulating reactions that could contribute to lung immune pathology (380, 508). The opposing immune effects that mycobacterial and helminth infections induce in the host, and even in the same tissue, are important to consider in light of either co-infection or prior exposure to either pathogen occurring. Such differences may cause a profound effect on the host's ability to control either pathogen; in one such example, prior mycobacterial infection was shown to abrogate immunity to *Nb* (509). Several studies investigating the effect of helminth infection on mycobacterial disease reveal the spectrum of effects that can be induced by different helminths, from detrimental (469, 472) to beneficial (450). These findings show that helminth infections can influence *Mtb*, but whether this influence is positive or negative depends on the context and species of helminth.

Maternal immunity is a critical determinant of infant survival especially in low-middle income countries, with various immunological factors such as cells, cytokines and antibodies transferred trans-placentally and via breast milk. The influence of maternal helminth infection on offspring immunity was introduced in Chapter 3; however, little data exists as to how maternal exposure to helminths may influence offspring immunity to *Mtb*. Helminth infection during pregnancy has been observed to alter offspring immunity in several ways. Helminth-specific CD4 T cells and IgG, IgG1, IgG2 and IgE antibodies have been detected in offspring born to helminth-infected mothers, transferred either *in utero*

or via breast milk, and often accompanied by Th1 and Th2 cytokine production by cord blood cells (456, 474, 510, 511). Maternal helminth infection can influence (negatively or positively) both antibody transfer and cytokine responses to *Mtb*-specific antigens (474, 512), BCG immunization (460) and even unrelated conditions such as allergic airway asthma (513). However, maternal helminth infection does not always influence infant vaccine-specific responses, as observed in a large Ugandan mother-infant cohort where infant tetanus and BCG-specific responses were unaffected by maternal helminth infection (458). These studies reveal that the effects of maternal helminth infection are not limited to helminth-specific responses, either in the mother or the offspring, but these effects may also be more complex than previously thought.

In order to provide further insight on the potential effects of maternal helminth infection on offspring immunity to unrelated pathogens and childhood vaccines currently of relevance in sub-Saharan Africa, we utilized murine models of hookworm and mycobacterial infection. These were used to investigate whether maternal *Nb* infection alters offspring immunity to *M. bovis* BCG infection, and whether it alters the effectiveness of BCG vaccination in the offspring. The preliminary data presented here show that helminth infection prior to pregnancy enhances offspring ability to respond to BCG vaccination and infection.

4.2 Materials and methods

4.2.1 Animal husbandry and ethics

All procedures performed using rodents were in accordance with protocols 011/018 and 012/054, as approved by the University of Cape Town Animal Research Ethics Committee.

Passage through Wistar rats was used to maintain the *Nb* life-cycle; these were ~150g at the time of infection. The rats were housed in open cages with continuous access to food and water. All related procedures were performed in the Biosafety Level 1 facilities of the University of Cape Town Animal Unit.

For experimental procedures, wild type BALB/c mice (males, females and offspring) were used. All animals were bred and housed in a specific pathogen-free environment (Animal Unit, University of Cape Town), and were housed in closed individually-ventilated cages with continuous access to food and water. All experiments were performed in a Biosafety Level 2 facility in accordance with the University of Cape Town's Health and Safety

guidelines. All experimental mice used were 2-15 weeks of age, and offspring used for the experimental outputs were age-matched. Mice were euthanized by halothane inhalation and death confirmed by cervical dislocation at the end of an experiment.

4.2.2 Mating and litter-swaps

Matings were set up as follows: 2 females of 7-8 weeks of age and 1 male were included per cage. The mice were housed together for 2 weeks, after which the male was removed. If successful, females gave birth to offspring 21 days post-fertilisation; cages were monitored daily to identify the birth of offspring. A maximum of 2 females and 8 offspring per mother were housed in each cage. For long-term experiments, offspring were weaned and separated from their mothers at 3 weeks of age; male and female offspring were housed separately.

4.2.3 *Nb* life-cycle

Nb is a rodent parasite that is used as a model of hookworm infection (514-516). The characteristic features of the life-cycle are outlined by Sotillo *et al* and Camberis *et al* (502, 515) and important steps are highlighted here. Helminth eggs are released into the soil as a component of faecal matter excreted by infected rodents. Once in the soil, the eggs hatch following an incubation period of approximately one week. The newly hatched worms then molt through two stages, L1 and L2, to become infective L3 larvae. L3 larvae penetrate the host's skin and enter the bloodstream. Larvae migrate to the lungs 1-2 days after entering the circulatory system, where they molt to the L4 stage of the life-cycle. These newly molted larvae are coughed up and swallowed, and continue migrating through the digestive system until they reach the small intestine (day 3-4 post-infection). There, the larvae molt one last time to become mature adult worms (L5) that can produce eggs, which are released in the faeces. Adult worms are cleared naturally by the host in immunocompetent mice by day 9 post-infection.

4.2.4 *Nb* infection, laboratory life-cycle and anti-helminth treatment

As previously mentioned, Wistar rats were used to maintain the laboratory *Nb* life-cycle to generate the infective larvae required for experimental procedures. L3 larvae were injected SC into the scruff of the neck (5 000 L3/rat in 0.5ml of 0.9% NaCl, 18-gauge needle). Faecal pellets were collected from days 6-8 post-infection. These were liquefied in 5µg/ml Amphotericin B before being plated out on moist filter paper in petri dishes at room temperature to facilitate egg hatching. One week post faeces collection, L3 larvae migrated

to the edges of the filter paper and were harvested for use in life cycle maintenance or experimental procedures.

For murine infections, L3 larvae were washed off the filter paper into 0.9% NaCl, and counted under a dissecting microscope to establish a concentration of 2500 L3/ml. Mice were infected SC in the skin of the abdominal cavity (500 L3/mouse in 0.2ml FS 1X PBS, 21-gauge needle). As previously mentioned, the mice would naturally clear the infection by approximately 9 days post-infection, but to ensure complete expulsion of the parasite, potential mothers were treated with 10µg/ml Ivermectin (Virbamec® LA) in their drinking water from days 7-14 post primary infection. The correct dose is approximately 4µg Ivermectin per 20g mouse per administration, which equates to 400µl per mouse of the drinking water containing 10µg/ml Ivermectin. To account for potential immune effects induced by Ivermectin treatment, helminth-uninfected mice were also treated with Ivermectin as described above.

4.2.5 *Mycobacterium bovis* BCG culture

The procedure for mycobacterial culture, infection and CFU measurement were adapted from methods outlined by Du Plessis *et al* (450). Stocks of BCG were kept at -80°C until required for experiments. One 100µl aliquot of frozen stock was inoculated into 20-40ml of liquid broth (Middlebrook 7H9 – Difco™) supplemented with OADC (Becton Dickinson) in a flask and incubated at 37°C for one-and-a-half weeks. The optical density reading of the culture was measured at a wavelength of 600nm, following de-clumping of the culture with a 26-gauge needle; bacteria were harvested in log phase, at an OD reading of 0.7-1.0. To maintain bacterial stocks, a portion of the culture was mixed with 10% glycerol, divided into aliquots and stored at -80°C. To confirm the integrity of the stocks and determine bacterial concentration, an aliquot was thawed 1-2 days after initial freezing, serially diluted and plated on 7H11 (Difco) agar culture plates supplemented with OADC. Following a three-week incubation, CFU counts were determined and recorded.

4.2.6 BCG vaccination and infection

Offspring were vaccinated IP at two weeks of age with 100µl BCG in FS 1X PBS (Danish strain 1331, Statens Serum Institut, Denmark) at a dose of 0.1mg/ml (0.01mg/pup, equivalent to dose-by-weight recommended for infants). Offspring used in control groups were given 100µl 1X PBS IP. Three weeks post-vaccination, PBS- and vaccine-treated mice were

infected IN with live BCG (cultured as described above) at a dose of $5x10^5$CFU/mouse in 50µl FS 1X PBS.

4.2.7 Sample collection and tissue processing

Three weeks after BCG infection, offspring were euthanized as described above, and tissues of interest harvested. The lungs, MST lymph nodes and spleen were removed by incision under sterile conditions for further analysis. Prior to processing, lung samples were incubated in digestion buffer (containing collagenase and DNase) at 37°C for one hour.

Once harvested, whole lung and spleen weights were recorded prior to further processing. To determine bacterial burden, segments of lung tissue were removed and weighed. Subsequently, the tissue segments were homogenized (Omni Prep® homogenizer, Omni-International) in sterile 1X PBS containing 0.05% Tween-80 (Sigma Aldrich). Once homogenized, serial dilutions of each sample were generated, and relevant dilutions plated onto Middlebrook 7H11 agar plates supplemented with OADC. Plates were incubated at 37°C for three weeks, after which plate CFU counts were performed and used to calculate CFU/ml for each sample.

The remaining tissue samples were processed in preparation for further analysis by flow cytometry. Tissue segments were homogenized in DMEM with 40 µM cell strainers to generate single cell suspensions. Once homogenized, red blood cells in lung and spleen samples were lysed with RCLB for 2 minutes, after which samples were centrifuged at 1200rpm for 5 minutes at 4°C to remove the lysis buffer. The cells were then washed and resuspended in DMEM. Viable cells in each sample were counted with a haemocytometer by Trypan Blue exclusion and counts used to determine total number of cells per organ. Cells were washed and resuspended in DMEM at a concentration of $5x10^6$ cells/ml, and were kept on ice until required.

All 4 experimental groups discussed in this chapter would be included in a single experiment and can be compared. For the sake of clarity in explanation, only 2 experimental groups were discussed at a time.

4.2.8 Cell staining for flow cytometry

Once processed and counted, cells were stained for analysis by flow cytometry as outlined below. For staining of extracellular markers, $1X10^6$ cells per sample were aliquoted into wells of a 96-well plate. The plate was centrifuged at 1200rpm for 5 minutes at 4°C to

remove excess medium, after which 25µl of the relevant antibody master mix was added to each well, and the plate incubated in the dark at 4°C for 20 minutes. Following staining, the plate was centrifuged as above and cells washed to remove unbound antibody. If flow cytometric analysis was performed immediately, cells were resuspended in 200µl MACS buffer and left in the dark at 4°C until required. If flow cytometric analysis was delayed, cells stained for the relevant cell-surface markers were fixed in 2% PFA in the dark at 4°C for 20 minutes. Following this, cells were washed and resuspended in MACS buffer, ready for acquisition, or permeabilization buffer to prepare cells for intracellular staining.

Prior to intracellular staining, cells were stimulated with PMA/ionomycin (20ng/ml and 1µg/ml respectively) and treated with GolgiStop™ (used according to manufacturer's instructions) at 37°C for 4 hours. Subsequently, cells were fixed as described above, and fixed cells were incubated in permeabilization buffer in the dark at 4°C for 45 minutes. Cells were then incubated in a blocking solution (containing rat serum and an FcγRII/III blocker) to prevent non-specific binding of antibodies for a further 10 minutes at 4°C in the dark. Next, 25µl of the primary antibody intracellular mix (or appropriate isotype control) was added to each well, and the plate incubated for 45-60 minutes at 4°C in the dark. Cells were then centrifuged as described above, and washed with 1X MACS buffer to prepare them for acquisition. **Table 4.1** details all antibodies used for intra- and extracellular staining.

Table 4.1. Antibodies used for flow cytometry.

Antibody for	Fluorochrome	Clone number	Company	Isotype control
CD4+ T cells				
CD3	A700	500A2	BD Pharmingen	N/A
	PE	500A2	BD Pharmingen	N/A
CD4	PerCP-Cy5.5	RM4-5	BD Pharmingen	N/A
	V450	RM4-5	BD Pharmingen	N/A
CD44	FITC	IM7	BD Pharmingen	N/A
IFNγ	APC	R4-6A2	BD Pharmingen	IgG1
IL-4	FITC	11B11	In-house	IgG1
B cells				
B220	V500	RA3-6B2	BD Pharmingen	N/A
MHC II	FITC	M5/114	In-house	N/A
	A700	2G9	BD Pharmingen	N/A
CD19	PerCP-Cy5.5	ID3	BD Pharmingen	N/A
CD21	APC	7G6	BD Pharmingen	N/A
CD23	PE	B3B4	BD Pharmingen	N/A

4.2.9 FACS procedure and gating strategies

Once appropriately stained, samples were acquired on a BD™ LSRFortessa flow cytometer, using FACSDiva™ software to record outputs. To account for spectral overlap between fluorochromes, single fluorochrome-labelled compensation bead tubes were prepared and acquired prior to sample acquisition. Following acquisition, data were analysed using the FlowJo® software package. The gating strategies used to identify cell populations of interest are presented below **(Fig. 4.1, 4.2)**. For intracellular staining, isotype controls were used to set the gates for positive cytokine staining.

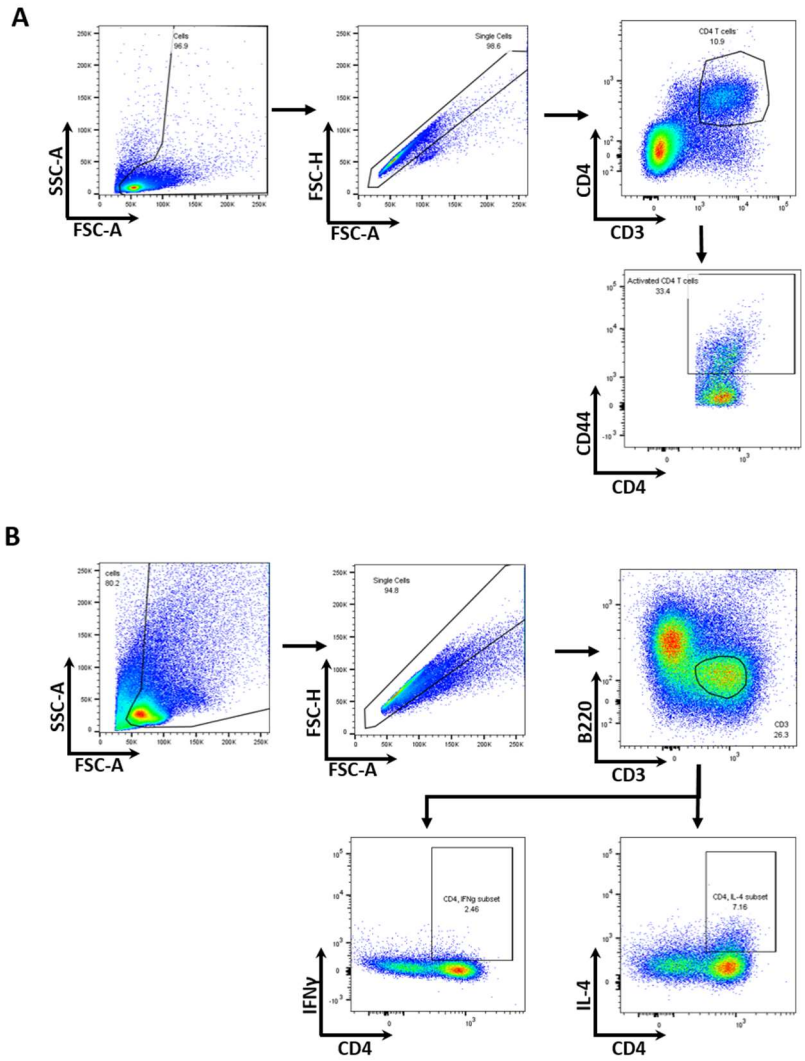

Figure 4.1. T cell flow cytometry gating strategy. Staining from spleen samples utilised to show T cell gating strategy **(A)**. Forward scatter (cell size) and side scatter (cell granularity) parameters are used to identify cells from the raw data. Within the lymphocyte population, helper T cells ($CD3^+CD4^+$) are identified and the proportion of activated CD4 T cells ($CD44^+$) determined. Staining from lung draining lymph node samples utilised to show intracellular staining for cytokines in CD4 T cells **(B)**. Cells are identified as in **(A)**, following which intracellular IFNγ and IL-4 expression of $CD4^+$ T cells are analysed.

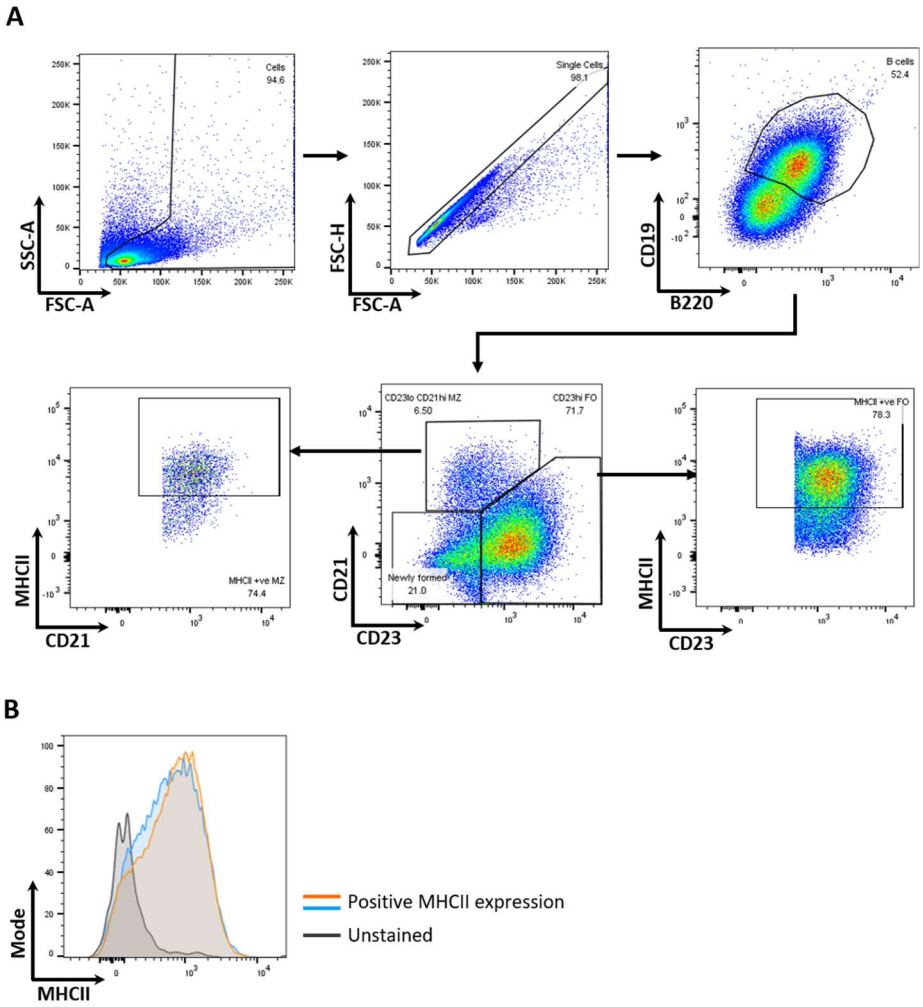

Figure 4.2. B cell flow cytometry gating strategy. Staining from spleen samples utilised to show gating strategy. Forward scatter (cell size) and side scatter (cell granularity) parameters are used to identify cells from the raw data **(A)**. Within the lymphocyte population, B cells($CD19^{+}B220^{+}$) are identified **(A)**. The B cell subpopulations are determined as follows: newly-formed (NF – $CD21^{-}CD23^{-}$), follicular (FO – $CD21^{lo\text{-}int}CD23^{hi}$) and marginal zone (MZ – $CD21^{hi}CD23^{lo}$) B cells, as well as the proportion of MHC II^{+} FO and MZ B cells **(A)**. Relative MHC II expression on FO and MZ B cells is also recorded **(B)**.

4.2.10 ELISA

The concentrations of antigens and antibodies described here were chosen specifically for use with this in-house ELISA assay. The procedure outlined below were adapted from similar methods described elsewhere (358, 517). BCG vaccine antigen was prepared as described in Chapter 2.

Nb *antigen*

Somatic Nb antigen was prepared from whole L3 larvae as described previously (456). Larvae were washed from the filter paper on which they are maintained into distilled water containing 50µg/ml P/S to kill larval-associated bacteria. Larvae were incubated in this

solution at room temperature for 1 hour, following which they were washed twice in distilled water and resuspended in 2ml distilled water. To disrupt cell walls, larvae were snap-frozen in liquid nitrogen and then homogenized in the distilled water. Following this, the homogenized solution was centrifuged to remove cellular debris. Once centrifuged, the soluble fraction was carefully decanted and the protein concentration in this fraction measured by BCA assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). Once the protein concentration was recorded, the soluble fraction was adjusted to a stock concentration of 500µg/ml and aliquots of the stock solution were frozen at -80°C until required.

Antigen-specific ELISA

Antigen-specific antibody ELISAs were performed. Coating antigens (BCG vaccine and Nb antigen) were used at 10µg/ml. Initial serum sample dilutions for the measurement of Nb-specific antibodies were 1:3, followed by a 1:3 serial dilution of that across a further 5 wells. Initial serum sample dilutions for the measurement of BCG-specific antibodies were 1:5, followed by a 1:5 serial dilution of that across a further 5 wells. The horse radish peroxidase (HRP)-linked IgG-specific secondary antibody was used at 1:5000, whereas the biotinylated IgG1 and IgG2a-specific antibodies were used at 1:2000. Streptavidin-HRP was used at 1:5000.

Nunc-Immuno™ MicroWell™ Maxisorp™ 96-well plates (Thermo Fisher Scientific; Waltham, MA) were coated with antigen and incubated at 37°C for 3 hours. Plates were washed 1X with PBS-Tween20 buffer and blocked with 200µl/well 2% BSA/1X PBS at 4°C overnight. Plates were washed 1X as above and sample added (50µl/well); plates were incubated at 4°C overnight. Plates were washed 3X as above and secondary antibody added (50µl/well); plates were incubated at 37°C for 3 hours. Prior to development, plates utilising biotinylated secondary antibodies were washed 3X as above and streptavidin-HRP (50µl/well) added. Plates were washed 3X as above and TMB substrate – prepared according to the manufacturer's instructions (TMB Microwell Peroxidase Substrate System, Roche Diagnostics GmbH, Mannheim, Germany) - added (50µl/well). Reactions were stopped with 0.5M H_2SO_4 and plates read on a Versamax™ 96-well plate reader (Molecular Devices; Sunnyvale, CA) at wavelength 450nm (540nm reference filter). Optical density readings (y-axis) vs. sample dilutions (x-axis) were plotted.

4.2.11 Statistical analysis

The Mann Whitney test was utilized for the analysis of unpaired two-group data. For the analysis of antibody data, individual dilutions were compared for significance with the Mann Whitney test. All analyses were two-tailed, and data are presented with the mean and standard deviation (SD). Significance was accepted at $p \leq 0.05$. GraphPad Prism software (v. 5.03) was used for all statistical analyses and graphs presented.

4.3 Results

4.3.1 Maternal helminth infection reduces lung bacterial burden following *M. bovis* BCG infection of offspring

Female BALB/c mice were infected with 500 L3 *Nb* larvae, and after 7 days the infection was cleared with a 5-day oral Ivermectin treatment. Once cleared, these mice were mated with male BALB/c mice. At 5 weeks of age, offspring were infected intranasally with *M. bovis* BCG. Once the infection had progressed for 3 weeks, offspring were killed and samples collected for further analysis **(Fig. 4.3A)**.

Offspring of *Nb*-exposed mothers exhibited a significant decrease in lung bacterial burden **(Fig. 4.3B)**. Importantly, these data show that maternal prenatal *Nb* exposure results in a clear protective effect against BCG infection in mature offspring and to the best of our knowledge, this is the first demonstration of such a finding.

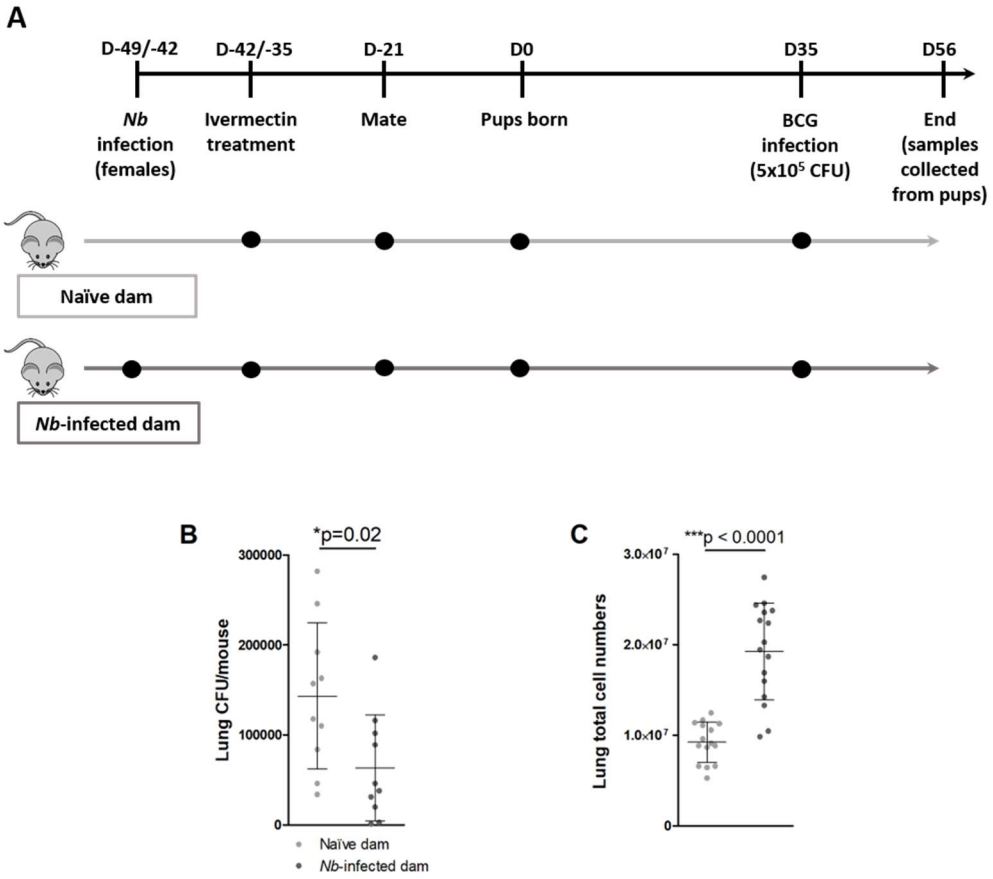

Figure 4.3. Maternal *Nb* infection results in reduced bacterial burden and increased cell infiltration in BCG-infected offspring. (A): BALB/c female mice were infected with 500 L3 *Nb* larvae, and 7 days later the infection was cleared with Ivermectin treatment. Once the infection had been cleared, mice were mated with BALB/c males. Pups were infected intranasally with BCG at 5 weeks of age. Samples were collected 3 weeks post BCG infection. Lung CFU/mouse values **(B)** and total cell numbers **(C)** were calculated. Data are pooled from 2 experiments (mean ± SD) with n=10-16 pups and n=4 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.2 Maternal helminth infection increases pulmonary cellularity, reflected by an upregulation of CD4 T cells, following *M. bovis* BCG infection of offspring

Associated with this protection was a significant increase in total lung cell numbers **(Fig. 4.3C)**, indicative of cellular expansion or infiltration. Analysis of lung T cell responses revealed no difference in the proportion of $CD4^+$ T cells between offspring of *Nb*-exposed vs. unexposed mothers, but $CD4^+$ T cell numbers were significantly upregulated in offspring of *Nb*-exposed mothers **(Fig. 4.4A)**, reflecting the upregulation observed with total lung cell numbers in **(Fig. 4.3C)**. Similar findings were observed upon investigation of $CD4^+$ T cell activation, with similar proportions of activated ($CD44^+$) $CD4^+$ T cells in both groups, but significantly increased cell numbers in offspring of *Nb*-exposed mothers **(Fig 4.4B)**.

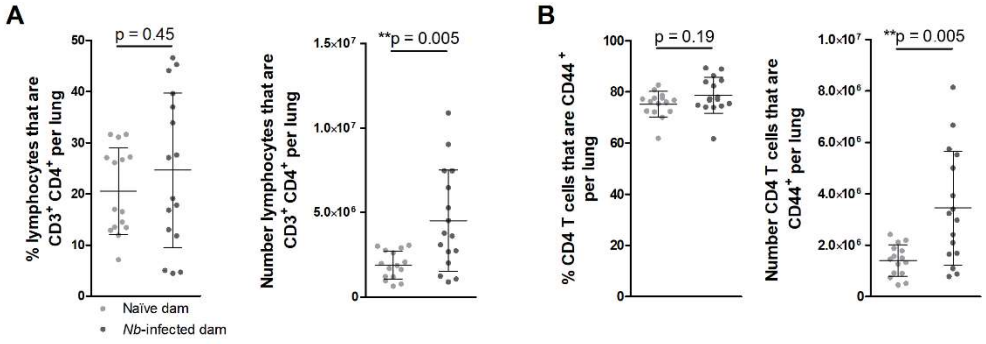

Figure 4.4. Maternal *Nb* infection is associated with increased numbers of activated Th cells in the lungs of BCG-infected offspring. Proportions and numbers of lung $CD4^+$ T cells **(A)** were calculated, following which activated ($CD3^+CD4^+CD44^+$) CD4 T cells were identified **(B)**. Data are pooled from 2 experiments (mean ± SD) with n=15-16 pups and n=4 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.3 Maternal helminth infection upregulates CD4 T cells in the lung draining lymph nodes following *M. bovis* BCG infection of offspring

Mediastinal lymph nodes (lung draining lymph nodes) were harvested and analysed. No significant difference in the total number of lymph node cells between experimental groups was found **(Fig. 4.5A)**. Similar to observations with lung tissue, no difference was found in the proportion of lymph node $CD4^+$ T cells, but offspring of *Nb*-exposed mothers exhibited significantly increased numbers of $CD4^+$ T cells as compared to offspring of unexposed mothers **(Fig. 4.5B)**. Offspring of *Nb*-exposed mothers also showed a significant increase in the proportion and number of activated $CD4^+$ T cells present in the lymph nodes as compared to offspring of *Nb*-unexposed mothers **(Fig. 4.5C)**. To observe whether maternal helminth infection caused skewing of $CD4^+$ T cell cytokine production to a type 1 or type 2 phenotype, IFNγ and IL-4 expression were measured. However, there was no difference in the proportion or number of $IFN\gamma^+$ or $IL\text{-}4^+$ $CD4^+$ T cells between experimental groups **(Fig. 4.5D)**.

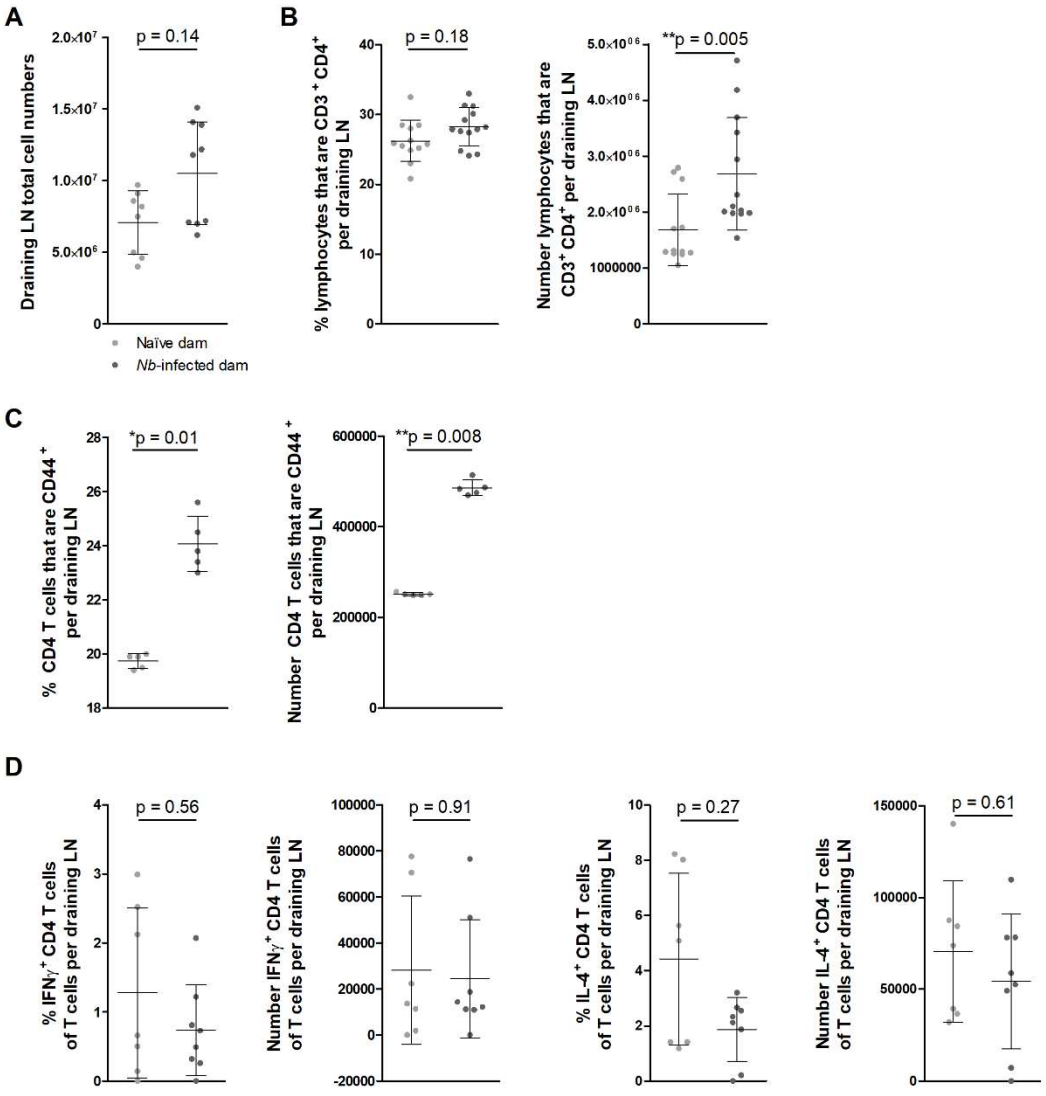

Figure 4.5. Maternal *Nb* infection is associated with an increase in activated Th cells in the lung draining lymph nodes of BCG-infected offspring. Total mediastinal lymph node cell numbers were calculated **(A)**. Proportions and numbers of draining lymph node $CD4^+$ T cells **(B)** were calculated, following which activated ($CD3^+CD4^+CD44^+$) CD4 T cells were identified **(C)**. Proportions and numbers of draining lymph node $CD4^+$ T cells producing IFNγ and IL-4 were calculated **(D)**. Data are pooled from 1-2 experiments (mean ± SD) with n=5-13 pups and n=2 mothers per group, for each experiment. Significance was assessed by the Mann Whitney test (*$p \leq 0.05$).

Lymph node B cell responses were also measured. Both the proportion and number of B cells were significantly increased in offspring of *Nb*-exposed mothers as compared to offspring of unexposed mothers **(Fig. 4.6A)**. Additionally, the relative expression of MHC II, a molecule associated with antigen presentation to $CD4^+$ T cells, was significantly upregulated on B cells from offspring of *Nb*-exposed mothers **(Fig. 4.6B)**.

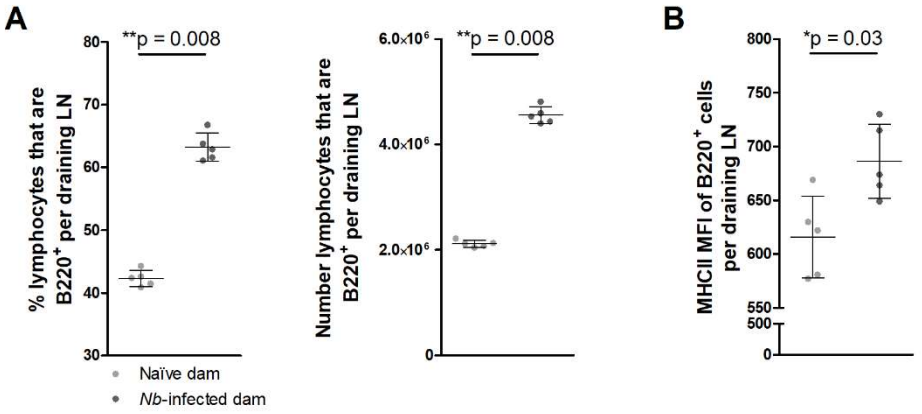

Figure 4.6. Maternal *Nb* infection is associated with an increase in lung draining lymph node B cells and their MHC II expression in BCG-infected offspring. The proportion and number of B cells in the draining lymph nodes were calculated **(A)**, as well as their expression of MHC II **(B)**. Data are representative of 1 experiment (mean ± SD) with n=5 pups and n=2 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.4 Maternal helminth infection modulates splenic B cell levels and MHC II expression following *M. bovis* BCG infection in offspring

The spleen represents another important site of adaptive immune responses to pathogens, and as such, splenic B cell responses were investigated. Although no difference was observed in spleen weight between offspring of *Nb*-exposed and unexposed mothers, total spleen cell number was significantly upregulated in offspring of *Nb*-exposed mothers **(Fig. 4.7A)**. A trend for an increased proportion of B cells in offspring of *Nb*-exposed mothers was observed, but this was not significant; however, these offspring did have a significant increase in B cell numbers, a difference reflective of total spleen cell numbers between groups **(Fig. 4.7B)**. Splenic B cells can be divided into 2 distinct subpopulations, namely follicular (FO) and innate-like marginal zone (MZ) B cells. These subpopulations are most commonly involved in responses to T cell-dependent and independent antigens, respectively (518, 519). Analysis of B cells by subpopulation revealed no difference in the proportion of follicular (FO) B cells between groups, but offspring of *Nb*-exposed mothers exhibited a significant increase in the proportion of marginal zone (MZ) B cells as compared to offspring of *Nb*-unexposed mothers **(Fig. 4.7C)**. However, the numbers of both FO and MZ B cells were significantly higher in offspring of *Nb*-exposed mothers **(Fig. 4.7C)**, again reflective of the difference in total spleen cell number. Investigation of MHC II expression on FO and MZ B cells revealed no difference in the proportions of MHC II^+ FO or MZ B cells, but both subpopulations had a significant increase in numbers of MHC II^+ cells in offspring

of *Nb*-exposed mothers **(Fig. 4.7D)**. However, there were no differences in the relative expression levels of MHC II between experimental groups for either FO or MZ B cells **(Fig. 4.7D)**, suggesting a limited effect of maternal helminth exposure on the antigen-presenting capabilities of spleen B cells during BCG infection.

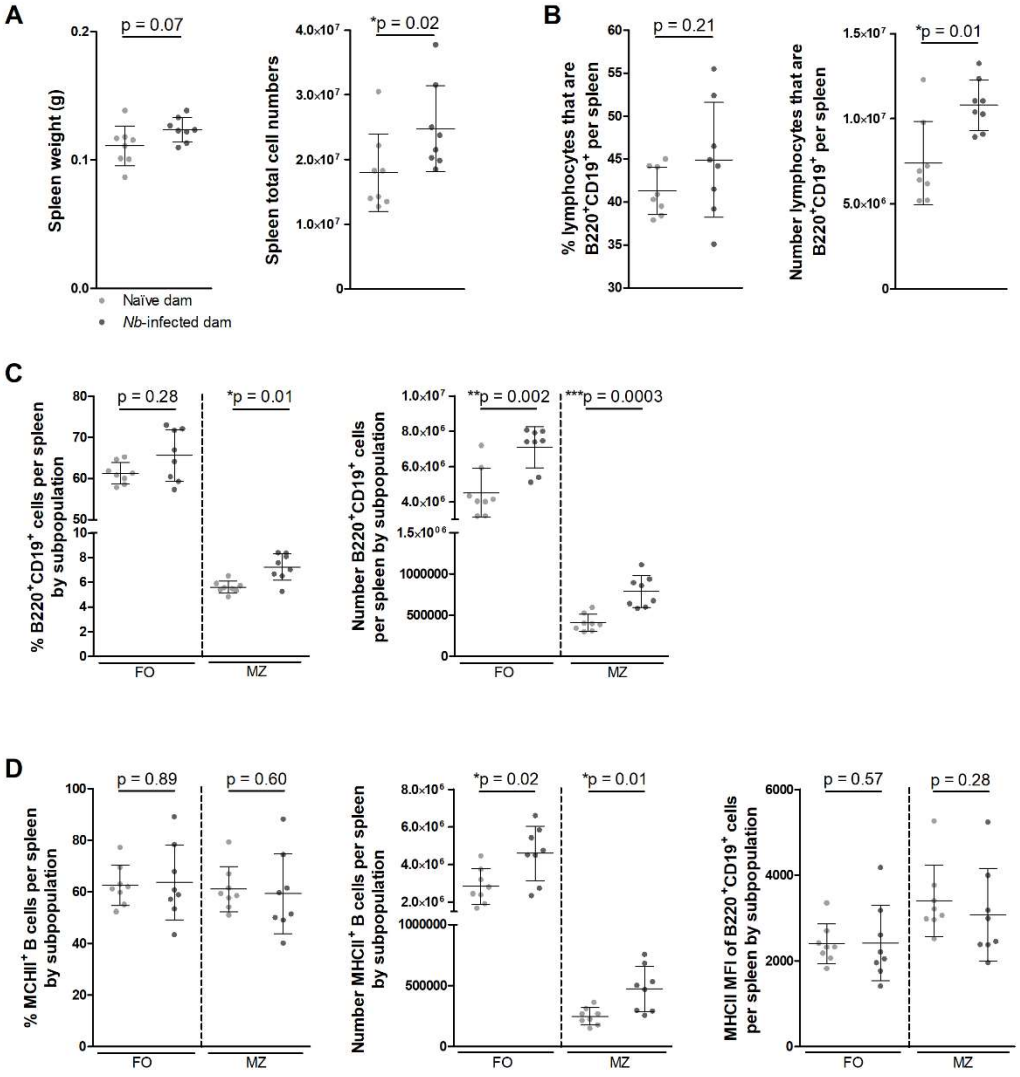

Figure 4.7. Maternal *Nb* infection is associated with increased numbers of splenic FO and MZ B cells expressing MHC II. Spleen weight and total cell numbers were calculated **(A)**. Splenic B cell proportions and numbers were calculated **(B)**, and stratified as ($CD23^{hi}CD21^{lo\text{-}int}$) FO and ($CD23^{lo}CD21^{hi}$) MZ B cells **(C)**. The proportion and number of FO and MZ cells expressing MHC II, as well as the mean fluorescence intensity of MHC II expression were calculated **(D)**. Data are representative of 1 experiment (mean ± SD) with n=8 pups and n=2 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.5 Maternal helminth infection upregulates *Nb* antigen-induced IgG1 levels in *M. bovis* BCG-infected offspring

To investigate whether the upregulation of B cells and their MHC II expression in offspring of *Nb*-exposed mothers translated to differences in antibody production, serum samples

were tested for the presence of antibodies to *Nb* somatic antigen and BCG vaccine antigen. Firstly, BCG-specific total IgG responses were measured to observe whether maternal *Nb* infection affected the vaccine-specific IgG response. Production of BCG-specific IgG was observed, but there was no difference in the IgG levels between experimental groups **(Fig. 4.8A)**. To determine whether there were differences in IgG subtype production, and if so, whether antibody production was skewed either to a Type 2 or a Type 1 phenotype, the subclasses IgG1 and IgG2a were investigated (520, 521). Additionally, although the mature offspring were helminth-unexposed, IgG subtype-specific responses to *Nb* antigen were measured to detect whether maternal *Nb* infection had induced any longer-lasting antibody responses. Offspring born to *Nb*-exposed mothers had significantly higher *Nb*-specific IgG1 levels in their serum than offspring born to naïve mothers, but no differences in BCG-specific IgG1 levels were observed **(Fig. 4.8B)**. There was no significant difference in *Nb*-specific IgG2a levels, but offspring born to *Nb*-exposed mothers showed a trend for increased BCG-specific IgG2a levels **(Fig. 4.8C)**.

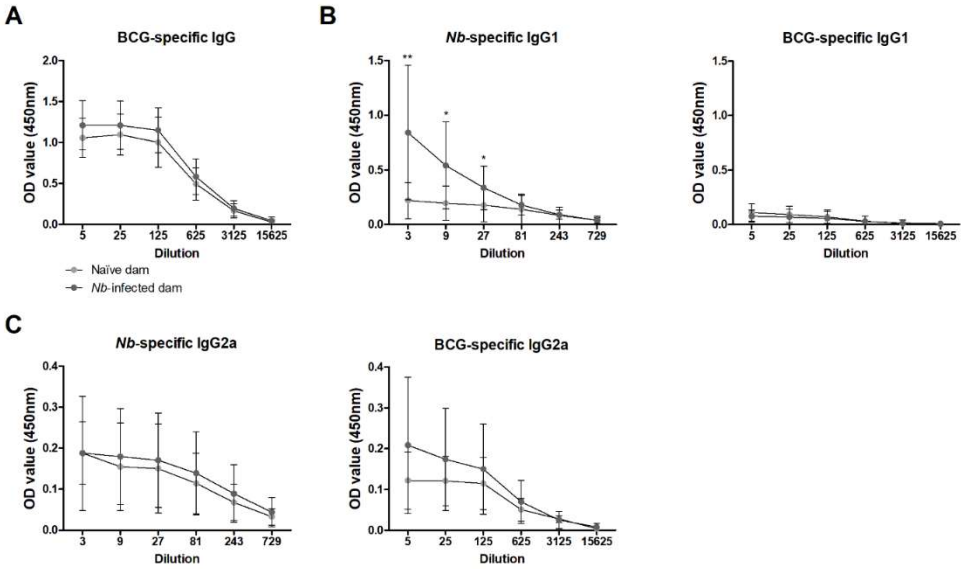

Figure 4.8. Maternal helminth infection results in significantly increased *Nb*-specific IgG1 in BCG-infected offspring. Serum BCG vaccine-specific IgG **(A)**, and *Nb*-specific and BCG vaccine-specific IgG1 **(B)** and IgG2a **(C)** levels were measured. Data are pooled from 2 experiments (mean ± SD) with n=12 pups and n=4 mothers per group. Significance at each dilution was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.6 The effect of maternal helminth infection on *M. bovis* BCG infection of offspring is less prominent following early-life BCG vaccination

Female BALB/c mice were infected with 500 L3 *Nb* larvae, and after 7 days the infection was cleared with a 5-day oral Ivermectin treatment. Once cleared, these mice were mated

with male BALB/c mice. At 2 weeks of age, offspring of these females were vaccinated with BCG, following which they were infected intranasally with *M. bovis* BCG 2 weeks after vaccination. Once the infection had progressed for 3 weeks, offspring were killed and samples collected for further analysis **(Fig. 4.9A)**.

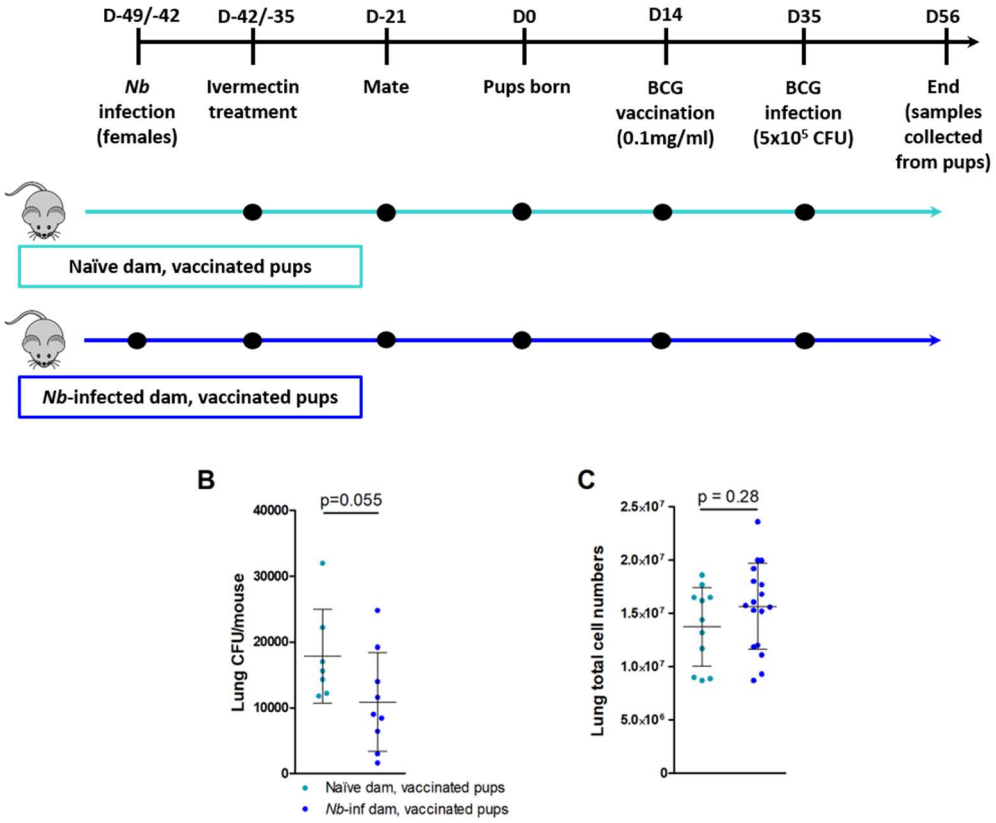

Figure 4.9. Maternal *Nb* infection is associated with decreased bacterial burden in BCG-vaccinated and infected offspring. (A): BALB/c female mice were infected with 500 L3 *Nb* larvae, and 7 days later the infection was cleared with Ivermectin treatment. Once the infection had been cleared, mice were mated with BALB/c males. Pups were vaccinated with BCG (or PBS control) 2 weeks after birth, and infected intranasally with BCG 3 weeks post BCG vaccination. Samples were collected 3 weeks post BCG infection. Lung CFU/mouse values **(B)** and total cell numbers **(C)** were calculated. Data are pooled from 2 experiments (mean ± SD) with n=7-17 pups and n=4 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

BCG vaccination administered to offspring of *Nb*-exposed mothers resulted in a near significant decrease in lung bacterial burden as compared to vaccination in the absence of maternal helminth infection **(Fig. 4.9B)**, but did not result in differing total lung cell numbers **(Fig. 4.9C)**. Analysis of lung $CD4^+$ T cell responses revealed no significant differences in either proportion or number of $CD4^+$ T cells between experimental groups **(Fig. 4.10A)**. Additionally, there were no differences in the proportion or number of

activated lung CD4⁺ T cells between vaccinated offspring of *Nb*-exposed or unexposed mothers **(Fig. 4.10B)**.

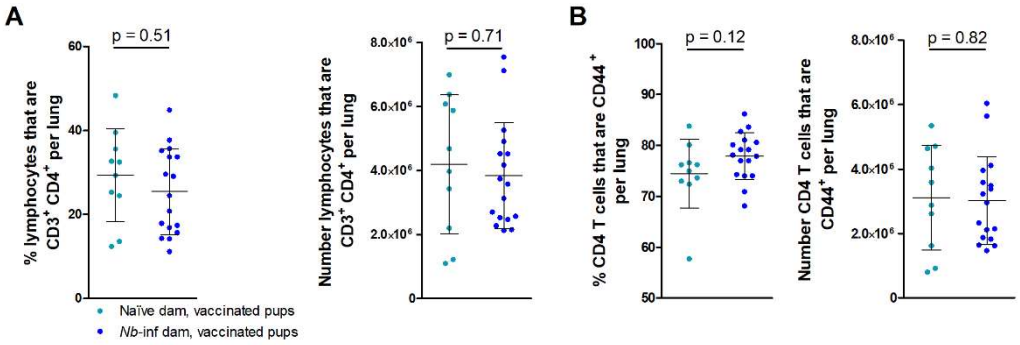

Figure 4.10. Maternal *Nb* infection does not associate with changes in lung Th cells in BCG-vaccinated and infected offspring. Proportions and numbers of lung CD4⁺ T cells **(A)** were calculated, following which activated (CD3⁺CD4⁺CD44⁺) CD4 T cells were identified **(B)**. Data are pooled from 2 experiments (mean ± SD) with n=10-17 pups and n=4 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.7 Maternal helminth infection upregulates activated CD4 T cells, IFNγ-expressing CD4 T cells and B cells in lung draining lymph nodes following BCG vaccination and infection of offspring

There were no differences in total draining lymph node cell number **(Fig. 4.11A)** nor in the proportion or number of CD4⁺ T cells between vaccinated offspring born to *Nb*-exposed/unexposed mothers **(Fig. 4.11B)**. However, vaccinated offspring of *Nb*-exposed mothers did exhibit significantly increased proportions and numbers of activated CD4⁺ T cells **(Fig. 4.11C)**. Additionally, vaccinated offspring of *Nb*-exposed mothers exhibited significantly increased proportions and numbers of IFNγ⁺ CD4⁺ T cells, but there was no difference in the levels of IL-4⁺ CD4⁺ T cells **(Fig. 4.11D)**.

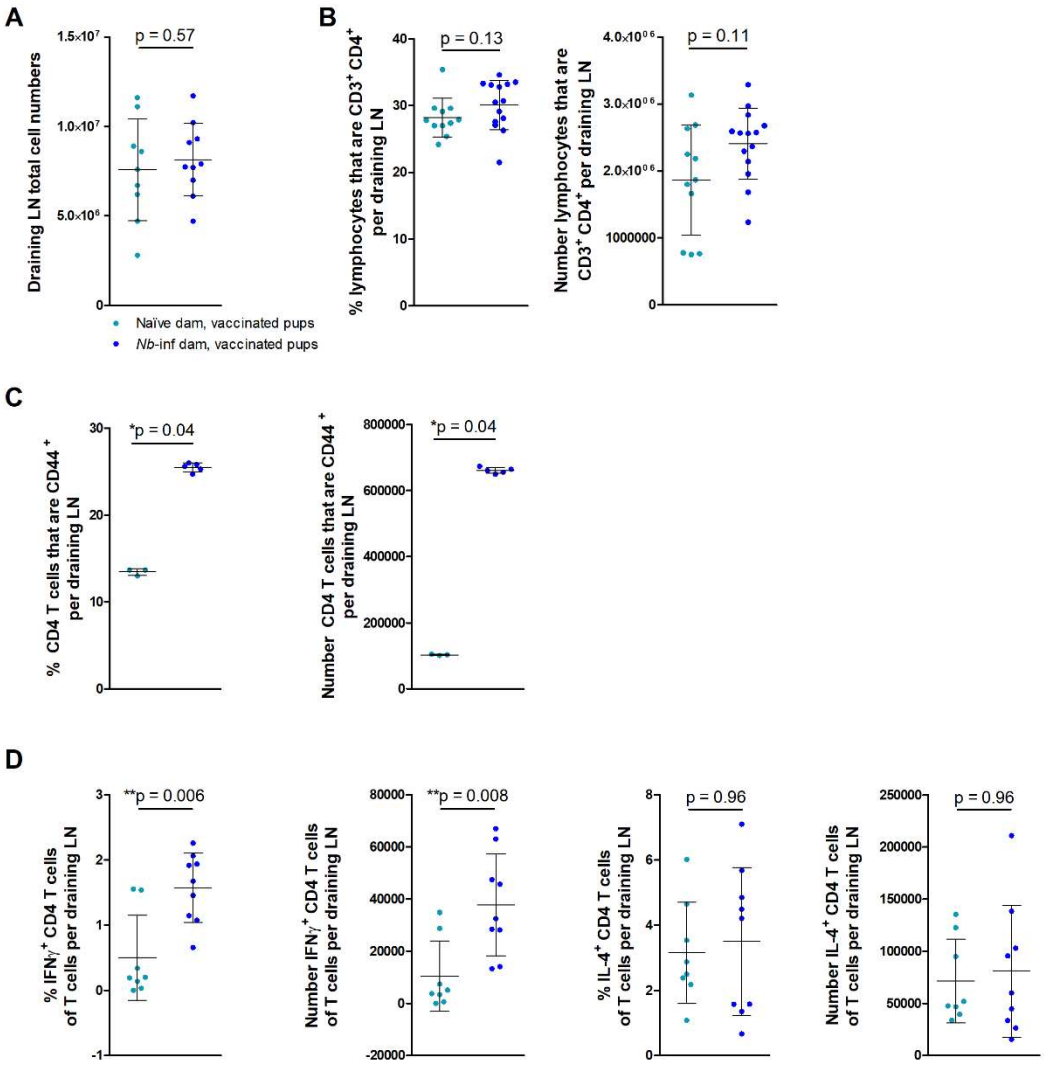

Figure 4.11. Maternal *Nb* infection is associated with an increase in activated Th cells and IFNγ-producing T cells in the lung draining lymph nodes of BCG-vaccinated and infected offspring. Total mediastinal lymph node cell numbers were calculated **(A)**. Proportions and numbers of draining lymph node $CD4^+$ T cells **(B)** were calculated, following which activated ($CD3^+CD4^+CD44^+$) CD4 T cells were identified **(C)**. Proportions and numbers of lung draining lymph node $CD4^+$ T cells producing IFNγ and IL-4 were calculated **(D)**. Data are pooled from 1-2 experiments (mean ± SD) with n=3-14 pups and n=2 mothers per group, for each experiment. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

Analysis of B cells in the draining lymph nodes revealed no difference in the proportion of B cells between experimental groups, but vaccinated offspring of *Nb*-exposed mothers did have significantly increased numbers of B cells **(Fig. 4.12A)**. The B cells from vaccinated offspring of *Nb*-exposed mothers also exhibited significantly reduced relative MHC II expression levels as compared to vaccinated offspring of *Nb*-unexposed mothers **(Fig. 4.12B)**.

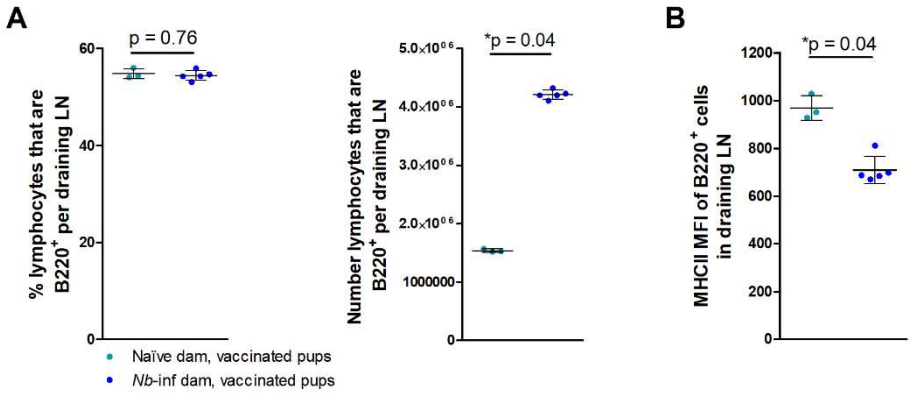

Figure 4.12. Maternal *Nb* infection is associated with an increase in lung draining lymph node B cells and decreased MHC II expression in BCG-infected offspring. The proportion and number of B cells in the draining lymph nodes were calculated **(A)**, as well as their expression of MHC II **(B)**. Data are representative of 1 experiment (mean ± SD) with n=3-5 pups and n=2 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.8 Maternal helminth infection modulates splenic B cell subpopulations of BCG-vaccinated and -infected offspring

There was no difference in either spleen weight or total cell number between experimental groups **(Fig. 4.13A)**. Investigation of splenic B cells revealed no difference in the proportions or numbers of B cells between experimental groups **(Fig. 4.13B)**. More detailed investigation of the B cell subpopulations revealed a significant increase in the proportion and number of MZ B cells in vaccinated offspring of *Nb*-exposed mothers, but no differences were observed with FO B cells **(Fig. 4.13C)**. Vaccinated offspring of *Nb*-exposed mothers exhibited a significant decrease in the proportions, but not numbers, of MHC II^+ FO and MZ B cells **(Fig. 4.13D)**. Analysis of the relative MHC II expression levels revealed significantly decreased MHC II expression on FO and MZ B cells from vaccinated offspring of *Nb*-exposed mothers **(Fig. 4.13D)**, reflecting the differences observed with proportions of MHC II^+ cells.

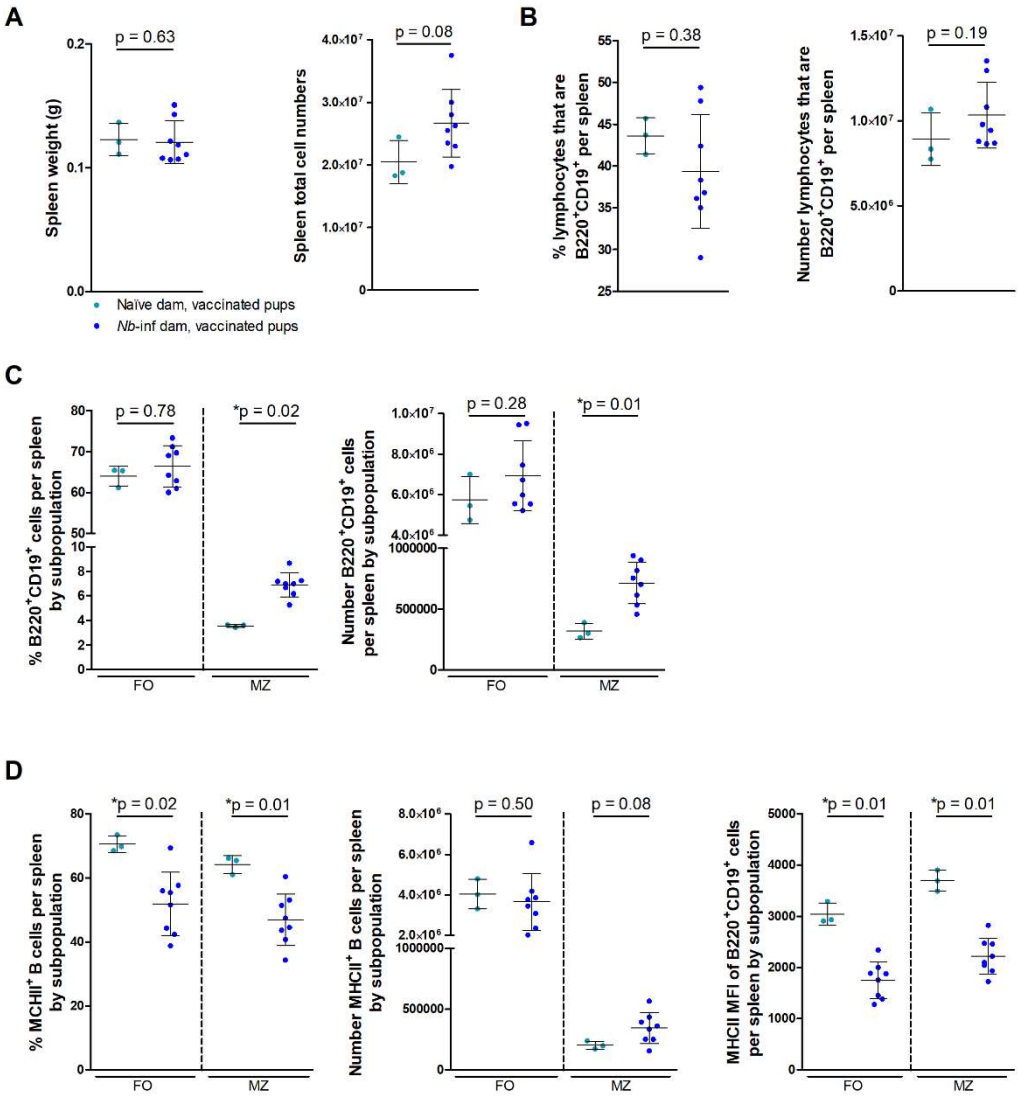

Figure 4.13. Maternal *Nb* infection is associated with increased splenic MZ B cells, but decreased MHC II expression on splenic B cells in BCG-vaccinated and infected offspring. Spleen weight and total cell numbers were calculated **(A)**. Splenic B cell proportions and numbers were calculated **(B)**, and stratified as ($CD23^{hi}CD21^{lo\text{-}int}$) follicular (FO) and ($CD23^{lo}CD21^{hi}$) marginal zone (MZ) B cells **(C)**. The proportion and number of FO and MZ cells expressing MHC II, as well as the mean fluorescence intensity of MHC II expression were calculated **(D)**. Data presented are representative of 1 experiment (mean ± SD) with n=3-8 pups and n=2 mothers per group. Significance was assessed by the Mann Whitney test (*p ≤ 0.05).

4.3.9 Maternal helminth infection modulates *Nb*-specific antibody levels in BCG vaccinated and infected offspring

As described previously, to investigate whether the differences observed with B cell populations in the lymph node and spleen translated into changes in their antibody production, serum samples were tested for the presence of *Nb*-specific and BCG-specific antibodies. Maternal helminth infection did not associate with differences in the levels of BCG-specific IgG between experimental groups **(Fig. 4.14A)**. Offspring of *Nb*-exposed

mothers had significantly increased levels of *Nb*-specific IgG1 **(Fig. 4.14B)**, but not of IgG2a **(Fig. 4.14 C)**. There was no difference in the levels of BCG-specific IgG1, but there was a trend for decreased BCG-specific IgG2a in offspring of *Nb*-exposed mothers **(Fig. 4.14B, C)**.

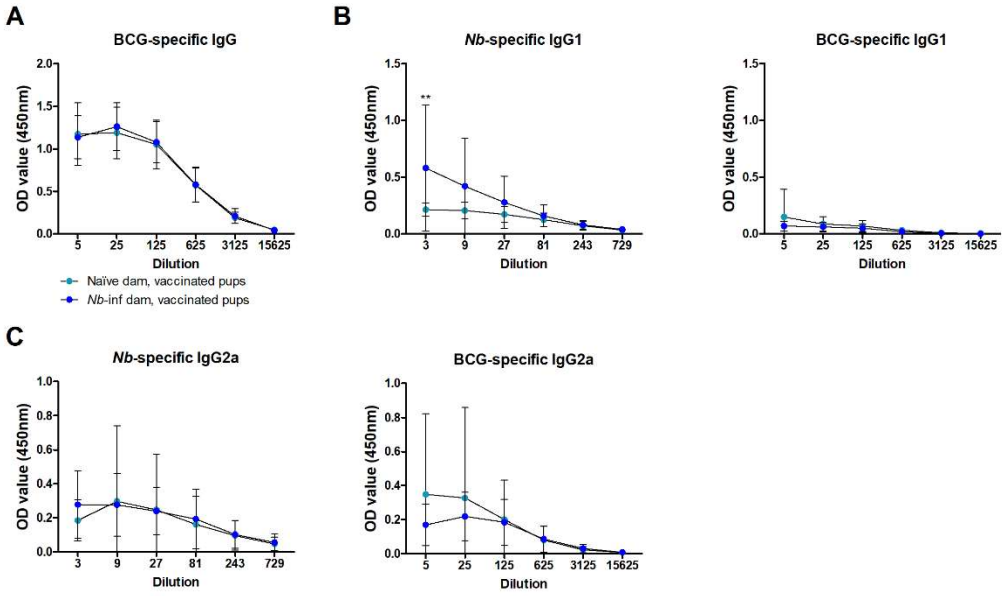

Figure 4.14. Maternal helminth infection results in significantly increased *Nb*-specific IgG1 in BCG-vaccinated and infected offspring. Serum BCG vaccine-specific IgG **(A)**, and *Nb*-specific and BCG vaccine-specific IgG1 **(B)** and IgG2a **(C)** levels were measured. Data are pooled from 2 experiments (mean ± SD) with n=11 pups and n=4 mothers per group. Significance at each dilution was assessed by the Mann Whitney test (*p ≤ 0.05).

4.4 Discussion

Observations from this *in vivo* model of maternal helminth and offspring bacterial infection reveal that maternal *Nb* infection alone can protect offspring against an intranasal BCG infection, most noticeably observed as a significant decrease in lung bacterial burden in the offspring of *Nb*-exposed mothers. Additionally, maternal *Nb* infection enhances cell recruitment to the site of infection, and may influence activation of the adaptive cell responses to BCG infection in the offspring. Investigation into the influence of maternal *Nb* infection on BCG vaccine effectiveness in offspring revealed a subtle synergistic effect, with the vaccinated offspring of *Nb*-exposed mothers exhibiting a near significant decrease in lung bacterial burden, and some upregulation of adaptive cell responses. Importantly, at the experimental endpoint for both BCG infection and BCG vaccination offspring were 8 weeks old, suggesting that the effects of maternal helminth infection were no longer being mediated through maternally-derived factors in the offspring. This could be indicative of fundamental immune changes induced in the offspring by maternal immunity.

Although our understanding of the influence of helminth exposure or infection on mycobacterial infection remains incomplete, the role of maternal helminth infection has been addressed in some studies. The upregulation of TB-specific antibody transfer (474) and cytokine responses to TB-specific antigens (512) suggests a potential protective effect of maternal helminth infection. Contradictory findings have also been reported, with children born to helminth-infected mothers exhibiting lower levels of IFNγ production in response to PPD (460). However, these studies do not address the impact of the altered immune responses on mycobacterial disease outcome. Varied effects of helminths on lung-associated pathology and bacterial burden have been reported. Chronic filarial infection in cotton rats didn't induce significant changes either in lung *Mtb* burden or *Mtb*-specific immune responses (470), and toxocariasis in mice yielded similar results (471). Conversely, infection with the trematode *Schistosoma mansoni* is suggested to have a more pronounced effect on mycobacterial infections, with increased bacterial burdens and dampened splenic responses reported (472, 522). Acute *Nb* pre-infection of mice was shown to have a transitory deleterious effect on control of lung *Mtb* burden, but is suggested to have a positive effect during BCG infection, with reduced bacterial burdens reported in co-infected mice (450, 469). Our finding of significantly decreased bacterial burden in offspring of *Nb*-exposed mothers lend support to the suggestion that helminth exposure or infection may provide protection against mycobacterial infection. The effects of helminth infection on lung inflammatory responses, indicative of cell infiltration, are just as varied. Either no difference in inflammation (469, 471), or an increase in inflammation are often reported (450, 472, 522). Our study suggests that even maternal helminth exposure can induce cellular infiltration in the lungs, potentially inducing lung inflammation. A similar study investigating maternal/offspring *Nb* infection alone did not reveal differences in pup total lung cell numbers (456); however these pups were investigated at a younger age and were not exposed to BCG.

It is well-known that opposing adaptive immune responses play a crucial role in the immune response to both helminth and mycobacterial infection. As such, it is important to consider the potential immune consequences of co-exposure to pathogens able to stimulate such different responses. It is generally accepted that helminth co-infection decreases CD4 T cell frequencies and responses to mycobacteria or mycobacterial-specific antigens, which could result in a dampened Th1 response in the lung (475, 523, 524). It has also been shown that

helminth co-infection can downregulate proliferation of PBMCs and spleen cells, as well as affecting their protective functions in response to stimulation with PPD (522, 525). However, such dampening effects do not always occur (470), nor are they always detrimental (524). We found a significant upregulation of overall $CD4^+$ and activated $CD4^+$ T cell numbers in the lung, with the increase in activated CD4 T cell numbers reflective of a co-infection study utilizing a model of *Nb* and BCG co-infection (450). Additionally, activated $CD4^+$ T cells in the lung draining lymph nodes were significantly increased, which is contrary to data suggesting dampened Th1 cell responses in the lymph nodes (524). Despite the increase in activated $CD4^+$ T cells, there was no associated skewing of cytokine expression by these cells. It is important to note that these are overall $CD4^+$ T cell responses, and may be specific for either pathogen. Additionally, cytokine analyses were performed with mitogen-stimulated cells alone and not unstimulated controls. As such, the values reported may be influenced to some degree by the presence of cytokines produced under homeostatic conditions. Although the cytokine milieu investigated here provides some insight to the immune environment under these experimental conditions, additional experiments with unstimulated controls would need to be performed to confirm whether these observed responses are specific to mitogen stimulation, or simply due to background cytokine production. Equally, as mitogen stimulation induces general cell activation, it would also be beneficial to investigate whether pathogen-specific antigens are able to induce cytokine production under these experimental conditions, or if maternal helminth exposure leaves the cytokine environment relatively undisturbed

B cells and the antibodies they produce have long been marked as important in helminth infection. As part of helminth-induced Th2 immunity, the production of IgA, IgG1, IgG4 and IgE have been reported; whether the antibodies are protective depends on the helminth studied, with IgE often identified as the subtype associated with protection (358, 384, 394, 526-528). However, alternative B cell functions may also be important in protective immune responses against helminths. B cell antigen presenting capabilities have been reported as important for protection against secondary *Nb* infection (358), and although not essential for *Nb* expulsion, B cells are important in expulsion of *H. polygyrus* (392). Conversely, the role of B cells and antibody in *Mtb* infection have long been under-appreciated, and have only recently been reinvestigated as an important component of anti-mycobacterial immunity (529). Various class-switched antibodies have been reported

as potentially protective against *Mtb* (305, 415). Additionally, patients with active TB disease or latent *Mtb* infection exhibited B cells with an atypical phenotype; treatment corrected this and increased T cell activity, highlighting the importance of healthy B cells in immunity to *Mtb* (530). Studies with B cell-deficient mice, on the other hand, yield conflicting findings, with both detrimental and beneficial roles for B cells against *Mtb* described (294, 531). Limited information is available regarding the role of B cells in helminth-*Mtb* co-infection, but it has been suggested that helminths are able to modulate mycobacterial-specific B cell responses, with potentially detrimental effects (532, 533). Our findings reveal that maternal *Nb* infection results in increased levels of B cells in lung draining lymph nodes and spleen, which contradicts the suggestion that helminth infection down-modulates mycobacterial-specific B cell responses (532, 533). However, it is important to note that a difference in helminth species and maternal infection vs. co-infection could be responsible for the differences observed. Additionally, the levels of MHC II expression on lymph node B cells and the number of MHC II^+ spleen B cells were increased in offspring of *Nb*-exposed mothers. Specific non-humoral functions of B cells, such as CD4 T cell co-stimulation and antigen presentation are important to consider in this context (358, 534). As mentioned, the necessity of the B cell's antigen presenting capabilities in protection against secondary *Nb* infection has been reported, with the presence of MHC II on the B cells being required (358). Thus, an upregulation of MHC II expression on B cells induced by maternal helminth infection may indicate an increased ability of these B cells to present antigen to CD4 T cells, thus boosting CD4 T cell responses to BCG infection. Our findings also reveal significantly increased levels of *Nb*-associated IgG1 in offspring of *Nb*-exposed mothers, along with moderate IgG2a production. *Nb*-associated antigens are known to induce IgG1 production, but not IgG2a (535). This points to either a helminth-associated or maternal immune component that is able to induce long-lasting IgG1 production in offspring without the offspring experiencing helminth infection, as the IgG1 antibody's half-life of 4-6 days indicates that no maternal antibody would still be present in the mature offspring (536). Importantly, IgG2a is a Th1-associated antibody not generally produced in a Th2 setting (520, 535). A possible explanation is that the IgG2a antibodies detected are specific for bacterial antigens. It has long been known that *Nb* requires a healthy gut microbiome to develop appropriately, indicating a close association between the helminth and bacteria (537). Although larvae are treated with antibiotic and washed

prior to processing for antigen, there may still be bacteria or bacterial antigens present in the homogenate, which would be able to induce antibody responses in the mouse. There was limited production of BCG-specific IgG1 in our study, and a trend for increased IgG2a in offspring of *Nb*-exposed mothers, which could suggest a mild beneficial effect of maternal helminth infection on the induction of Th1-associated antibodies to BCG. Another plausible explanation for the presence of long-lived helminth-specific responses in helminth-naïve offspring is maternal microchimerism. This a process during which maternally-derived cells are transferred to and tolerated by offspring and which can influence offspring immune responses (538, 539). The transfer of maternal helminth-elicited CD4 T cells to offspring has been observed and implicated in protective responses to subsequent helminth infection in the offspring (456), suggesting that maternal microchimerism may play an important role in the development of long-term immunity in infants. The data presented here support this, as the offspring investigated were weaned and fully grown, removing the possibility that the effects observed were purely due to passive maternal immunity.

BCG vaccination is known to induce protective responses to *Mtb* infection in mice, characterized by decreased lung and spleen bacterial burdens and lung pathology, as well as increased Th1-specific responses (e.g. enhanced IFNγ production) (497, 540). Several studies have investigated whether helminth infection can alter these protective responses. Certain helminths are able to modulate protective responses induced by BCG vaccination, with increased bacterial burden noted in a murine model and decreased IFNγ responses observed in murine and clinical studies (466, 467). However, the effects appear to be species-specific, as not all helminths elicit the same effect (468). Similarly, the effect of maternal helminth infection on BCG-associated responses are also species-specific, as reports range from potentially detrimental to potentially beneficial (458, 460, 512). The findings from our study suggest that the effect of maternal helminth infection on BCG vaccination may be more subtle than that observed with either mycobacterial infection or helminth-mycobacterial co-infection. A trend for decreased bacterial burdens in vaccinated offspring of *Nb*-exposed mothers mimicked the finding with unvaccinated offspring from *Nb*-exposed mothers; however, there were no differences in total lung cell number or lung-specific CD4 T cell populations. As observed with unvaccinated offspring from *Nb*-exposed mothers, lung draining lymph node activated CD4 T cells were significantly increased in

vaccinated offspring from *Nb*-exposed mothers. Additionally, IFNγ$^+$ but not IL-4$^+$ CD4 T cells were significantly upregulated in vaccinated offspring from *Nb*-exposed mothers, which is in agreement with prior research showing a potential positive effect of maternal helminth infection on mycobacterial cytokine responses (512). Although the majority of T cell findings mimic our observations with maternal helminth infection and BCG infection, the effect of helminth exposure was decreased.

It is well-known that BCG induces antibody production, with IgG, and the subtypes IgG1, IgG2 and IgG3 (Th1-type antibodies) as the most prominently produced subtypes (295, 428). More recently, it has also been observed that BCG vaccination elicits long-lived memory B cells that respond well to PPD stimulation (541). However, it remains unclear how helminths may affect B cell responses to BCG vaccination. As observed with the T cell responses, the effect of maternal helminth infection on B cells following BCG vaccination was not as pronounced as the effect on BCG infection alone. Lymph node B cell numbers were upregulated, as were MZ B cells, but no differences were observed with overall spleen B cells or the spleen FO cell subpopulation. Interestingly, vaccinated offspring from *Nb*-exposed mothers exhibited decreased MHC II expression on lymph node B cells, and decreased proportions of MHC II$^+$ and MHC II expression levels on spleen B cells, which was not observed with maternal helminth-BCG infection alone. This could indicate a decrease in the antigen presenting capacity of these cells. There was also a significant increase in *Nb*-associated IgG1 levels and a trend for decreased BCG-specific IgG2a levels in vaccinated offspring of *Nb*-exposed mothers. It has been shown that although not substantial, BCG vaccination is able to induce both IgG1 and IgG2a production, but that the antibodies are more readily detectable from 8 weeks post infection (542-544). This provides an explanation for the low levels of BCG-specific antibodies in this study, as offspring were euthanized 6 weeks post BCG vaccination. Alternatively, the difference in overall IgG and levels of IgG1 and IgG2a suggest contribution by other antibody subtypes, for example IgG2b. The trend for decreased IgG2a in offspring of *Nb*-exposed mothers suggests a moderate down-regulation of Th1-associated antibodies by *Nb* exposure. However, these effects do not appear to influence the effectiveness of BCG vaccination, as attested to by the reduction in bacterial burden in offspring of *Nb*-exposed mothers.

Taken together, these findings reveal that modulation of immune responses is not limited to concurrent helminth infection, but that maternal helminth infection/exposure can

influence her offspring's response to antigens unrelated to the helminth in question. Although the effect is subtle, it may reveal a more potent modulatory effect induced by *Nb*, as analyses were performed in offspring that had never experienced a helminth infection. Additionally, our data suggest that maternal helminth infection may act synergistically with BCG vaccination to enhance immune responses that are protective against a live BCG infection. These data highlight the fact that helminth exposure may be able to influence mycobacterial infection and BCG vaccination in subtle but complex ways, and that its effects are not limited to the site of BCG infection. These findings also lend support to the potential influence of helminth exposure on antibody responses to childhood vaccines as discussed in Chapter 3, and are in agreement with our finding that although an association exists between helminth and vaccine antibody responses, helminth exposure does not appear to have more than a mild effect on vaccine efficacy. An important consideration is that, although there are some parallels, there are several differences in the findings between this chapter and Chapter 3, highlighting the importance of careful comparison within translational research. A variety of factors could be responsible for these differences, for example the use of peripheral blood (clinical) versus lung tissue (murine model), samples which explore different compartments of *Mtb* immune responses. Additionally, the study of *Mtb* versus BCG and the use of different helminth types could yield varying results. These differences in study design are important to consider for future work; nevertheless, the findings from this study provide an insightful starting point.

A caveat of this exploratory study is that certain findings are only representative of one experiment, and as such these experiments would need to be repeated and findings confirmed prior to firm conclusions being drawn. However, the compelling evidence for a protective effect of maternal helminth infection on BCG infection and vaccination, as discussed, warrants further study.

Chapter 5: Concluding remarks and future work

5.1 Summary of findings

In our clinical study, which used humoral responses as an indicator of immune responses to various antigens and pathogens, we found that total non-specific plasma IgG responses were significantly lower in children who acquired an *Mtb* infection, suggesting that higher total IgG responses may be associated with a decreased risk of *Mtb* infection. The same association was observed with certain vaccine responses, as a significant increase in measles-specific IgG and a trend for increased BCG-specific IgG2 responses were observed in QFT- infants. The measles and BCG vaccines have been associated with a reduction in all-cause mortality (417, 418), and BCG vaccination is known to provide partial protection (202) and may enhance anti-*Mtb* protective responses (297, 496, 497). In light of this, our findings could be attributed, at least in part, to vaccination with these childhood vaccines. However, as the measles and BCG-specific responses did not correlate with the total IgG responses, it is important to consider that other components of this non-specific IgG response could play a role in protection against *Mtb* infection.

The geographical overlap of tuberculosis and helminth infections in the study region (330, 408) led us to investigate the potential influence of helminth infection on *Mtb*-associated outcomes. Appropriate water sources and sanitation are effective control measures against helminth infection (482, 483), and available to most participants in this study along with anti-helminthic treatment (484). Despite this, helminth infections do still occur, as the presence of class-switched helminth-specific antibodies indicate. However, this helminth exposure does not affect the risk of acquiring an *Mtb* infection or IFNγ production by *Mtb*-specific T cells. Helminth-specific antibody responses relate positively to vaccine-specific antibody responses, but this association does not appear to translate to any wider outcomes measured in this study, suggesting a modest influence of helminth exposure on responses to childhood vaccines.

A more in-depth investigation of the interaction between helminth exposure and mycobacterial infection in a murine model revealed that maternal helminth exposure protected offspring against *M. bovis* BCG, an unrelated pathogen. Additionally, it may enhance protective responses to mycobacteria induced by BCG vaccination. Our findings suggest that these protective effects are mediated through the induction of protective

adaptive cell responses in the secondary lymphoid tissues. Interestingly, the antigen-presenting function of B cells rather than their antibody production may be more important in these responses. Importantly, the presence of helminth-specific antibodies in helminth-naïve adult offspring of helminth-exposed mothers suggests a longer-term impact of maternal immunity on infant immune development than previously thought. This is supported by prior studies in our research group, where maternal helminth infection was shown to increase innate and adaptive cell populations, as well as induce early germinal centre development (456, 545), responses one would only expect to occur following exposure to a foreign antigen or pathogen. This suggests that one or more components transferred from mother to offspring do not only confer passive protection, but can actively induce the development of long-lasting protective responses in the offspring. The protective effect of maternal helminth exposure on BCG infection was less pronounced if a BCG vaccine was administered prior to BCG infection in the offspring. This suggests that the high level of control induced by BCG vaccination partially masks any protective effect the maternal helminth exposure may have. Albeit subtle, maternal helminth exposure was still able to upregulate cellular responses in the offspring secondary lymphoid tissues, suggesting a synergistic effect of maternal helminth exposure and BCG vaccination in protecting offspring against a subsequent BCG infection.

Taken together, our findings suggest that the induction of heterologous protective adaptive immune responses in children, through vaccination or maternal helminth infection history, could provide protection against childhood mycobacterial infection and may enhance vaccine effectiveness.

5.2 Caveats, limitations and future work

Various factors contribute to the robust nature of this research. The clinical research component had both longitudinal and cross-generational components, two factors which aid in our understanding of longer-term effects on infant immunity. Additionally, our broad analysis of both vaccine and helminth humoral responses and how these relate to *Mtb* infection susceptibility provide a wider view of the interplay between the responses to various immune challenges and how these relate to susceptibility to a largely unrelated pathogen. Moreover, the use of a murine model to investigate mechanisms potentially underlying observations made with our clinical cohort, adds a translational element to our research. This approach provides an increased scope for insights which may not be possible

with either clinical or animal research alone. However, as with any study, there are certain caveats and limitations to the research presented here which would be important to address in future work; these are discussed below.

Our clinical cohort was a sub-study that was part of a larger parent trial, as described in Chapter 2 (materials and methods section). The resulting sample set that was available for testing and analysis was, therefore, only a fraction of the entire cohort. A greater sample number could have leant more power to the findings presented here, and may have allowed for a parallel analysis in those infants who subsequently developed active TB disease, an approach that could not be utilised currently due to low numbers of active TB disease cases in the sub-study. Unfortunately, this cannot be addressed with the current cohort, as a limited number of infants were enrolled to this sub-study, and the parent trial has already been concluded and as such, no more enrollments are possible. As the mothers were not the focus of the trial, the information collected from them via questionnaire was limited. This resulted in insufficient data on maternal *Mtb* infection status and feeding data (e.g. breastfed vs. bottle-fed), factors whose potential influence on infant immune responses to *Mtb* could be important to consider, as maternal immune history and infant nutrition do influence immune development (discussed in detail in Chapter 1, sections 1.2.1 and 1.2.2, respectively). Infants enrolled in this study had ready access to good sanitation, clean water and regular deworming treatments. Subsequently, no current *A. lumbricoides* or *T. trichiura* infections were detected in the infants, and as such we were limited to analyzing the effect of helminth exposure rather than current infection on the infants' immune responses, where current helminth infection may have had a more pronounced effect. A more detailed analysis would require samples from a cohort located in another helminth-endemic region, where appropriate sanitation, clean water and deworming are not as readily accessible. Alternatively, a more stringent study design with an emphasis on the recruitment of infants with active helminth infection could be informative in assessing the role of current helminth infection on the outcomes discussed.

Within the clinical study, our focus on in-depth analysis of the infants' samples and clinical characteristics confined our analysis of maternal samples, as did the limited data available for the mothers. Despite this, it would be interesting to test a greater number of maternal samples, and to increase the breadth of antibody responses tested, to provide a more detailed description of the association between maternal and infant humoral responses in

this cohort. Additionally, as maternal samples were expected to exhibit an antibody response of greater magnitude than infant samples, different sample starting dilutions were used; however, a more accurate comparison would be achieved if the same starting dilution were used, a method that will be employed in future.

There is increasing interest in the role of *Mtb*-specific humoral immune responses in protection against *Mtb* (285, 305). This, paired with the fact that we have not yet identified the protective component of the total IgG response, point to an analysis of antibody responses to *Mtb*-specific antigens as an important subsequent step. There is some indication of pertinent antigens to investigate such as Ag85A or ESAT-6, (289, 307), or the capsular components AM/LAM (286, 287, 302), with antibodies specific for these antigens suggested to be involved in protective anti-*Mtb* responses. Thus, analysis of antibody responses to these antigens would be a good starting point.

Thus far, this clinical study represents a phenotypic analysis of immune responses to the various antigens and pathogens. However, a prominent caveat is the absence of functional assays, which would investigate the mechanisms underlying the observations we have made. Such functional studies could identify antibody-mediated processes involved in protective responses to mycobacterial infection in this cohort, providing further insight into host immune responses that could be targeted by new treatments and vaccines. Several studies have shown that mycobacterial-specific antibodies can enhance cell-mediated protective responses, often by enhancing macrophage-associated effector functions (296, 300, 305). We have begun optimizing an opsonophagocytosis assay to assess the functional response of antibodies already measured in the serum samples, to test whether the protective effect observed translates into an enhanced ability to control mycobacterial infection. The assay we have employed uses a human macrophage-like cell line and mycobacteria labelled with pHrodo^{TM}, a dye which fluoresces at low pH and is used to measure bacterial uptake and killing by phagocytosis (detailed methods can be found in Chapter 6 – Appendices). Preliminary findings show that plasma from QFT- infants may have an enhanced capability to induce phagocytosis as compared to QFT+ infants, as indicated by the greater proportion of dye^{+} cells **(Fig 5A)**. This was not due to differing activation levels of the stimulated cells, as the cells all expressed equivalent levels of CD11b, a marker that is known to be expressed on differentiated THP-1 cells (546) **(Fig. 5B)**. Although this is promising, the optimization would need to be completed and more

samples analysed to confirm whether this finding is valid. A confirmatory procedure that could also be used is the measurement of phagocytic uptake of GFP-expressing bacteria by flow cytometry and confocal microscopy to confirm uptake and co-localisation with the phagolysosome as a proxy of intracellular killing.

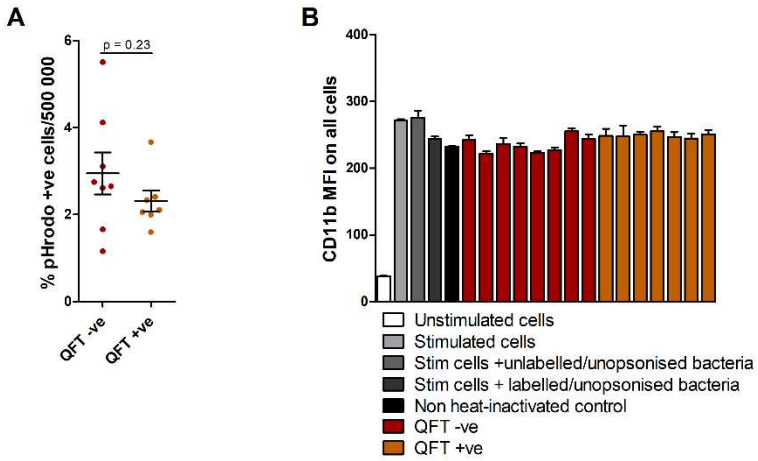

Figure 5. Higher antibody levels from QFT- infants may enhance phagocytosis of mycobacteria. Auxotroph *Mtb* bacteria were stained with pHrodo™ and opsonised with heat-inactivated plasma from QFT- and QFT+ infants, following which phagocytosis by differentiated THP-1 cells was allowed to proceed. Cells were subsequently analysed for pHrodo™ positivity **(A)** and CD11b expression **(B)**. Comparison in **(A)** was assessed for significance by the Mann Whitney test, and comparisons in **(B)** by the Kruskal-Wallis test ($p \leq 0.05$).

The promising results from our murine model provide a good starting point and strong basis for future work. Certain experiments need to be repeated to provide confirmation of the results, but the clear findings already obtained, such as bacterial burdens, reveal that these experiments are worth pursuing and more in-depth experiments to investigate certain components of the immune responses involved, e.g. the role of B cell antigen presentation, could prove to be insightful. Due to time and sample constraints, analysis of cytokine responses was limited. However, this would be an important analysis to extend, and it would be beneficial to include other pertinent cytokines such as IL-12 and TNF-α (type 1), and IL-5 and IL-13 (type 2). It could also prove insightful to measure levels of cytokines produced in addition to analyzing the proportions of CD4 T cells producing each cytokine, as presented here. With the model as is, an in-depth analysis of alveolar macrophage populations would also be interesting, as these are some of the first cells encountered by *Mtb* in the lung (217), and it would be important to investigate changes in lung pathology via histology.

The findings obtained thus far provide the basis for several potential avenues of investigation which could be followed. The longevity of increased cell activation and presence of helminth-specific antibody in offspring long after they have been weaned suggests that the influence of maternal immunity is not merely passive. Whether these upregulated responses are due to general immune activation or due to stimulation by cross-reactive molecules, e.g. antigens, remains to be tested. An initial approach to this could be an investigation of the antigen specificity of T and B cells, to observe whether they are helminth or *Mycobacterium*-specific, or if they are specific for an epitope allowing for cross-reactivity between these organisms. Additionally, certain important maternal immune components could be neutralized and the effect on offspring responses to BCG measured. For example, an investigation of the influence of maternal helminth infection on offspring helminth-specific immune responses revealed that maternal memory CD4 T cells were transferred to pups via breastmilk and that they may be involved in protection against helminth infection in the pups (456). Neutralisation of such transferred cells in offspring prior to BCG vaccination and/or infection could reveal whether offspring are more susceptible without the protection afforded by those cells, whatever their antigen specificity. It would also be interesting to investigate whether helminth during pregnancy alters any of these findings. Although anti-helminthic treatment during pregnancy does not appear to have a substantial effect on infant cytokine responses as measured in blood samples (462), immune effects induced by helminth infection during pregnancy may have more subtle effects than can be detected with blood samples alone, suggesting that an *in vivo* model may provide more insight. Additionally, due to the complexity of immune responses induced by different helminth species (outlined in Chapter 1- section 1.5.2), it would be beneficial to determine whether these same protective effects are observed with helminth species other than *N. brasiliensis*.

Although some confirmatory experiments would need to be performed to substantiate this research, the data presented here are promising. Our findings highlight the importance of heterologous immune activation, which could provide another pathway to boost general immunity. Additionally, the significant protective effect that helminth exposure or infection could provide against mycobacterial infection exhibits that helminths are not always detrimental to the immune control of other pathogens and can, in fact, be beneficial. These surprising findings highlight the benefit of novel approaches to the study of pathogens such

as *Mtb* that remain a global challenge, as they may provide new insights into unusual mechanisms that could be employed in the control of these pathogens, such as heterologous immune responses or helminth exposure.

Chapter 6: Appendices

6.1 Supplementary methods

6.1.1 Opsonophagocytosis assay

6.1.1.1 THP-1 cell culture

For *in vitro* investigations of antibody function, a human monocyte cell line, THP-1, was kindly provided by Dr. Georgia Schäfer; the cell line can also be acquired from ATCC® (TIB-202™). Cells were maintained at 37°C in 5% CO_2 in RPMI-1640 medium containing 2mM L-glutamine (Gibco®, Thermo Fisher Scientific); 10% FBS was added to generate complete medium, and a penicillin/streptomycin mix was added to prevent bacterial contamination. Cultures were maintained at a density of $1X10^5$-$1X10^6$cells/ml in 75cm^3 flasks and media was replaced as necessary.

To ensure availability of cells for all experiments, aliquots of cells were periodically frozen. Each 1ml aliquot contained $1X10^6$ cells in a mixture of 90% FBS/10% DMSO. Cells were frozen slowly overnight at -80°C, after which they were transferred to long-term liquid nitrogen storage. When required, an aliquot of cells was removed from storage, rapidly thawed at 37°C and immediately transferred to pre-warmed media. Cells were centrifuged to remove DMSO and resuspended in fresh medium prior to incubation.

To generate macrophage-like cells that could be used for phagocytosis assays, THP-1 cells were differentiated in RPMI-1640 containing 100nM PMA. Cells were left to differentiate for 3 days (37°C in 5% CO_2), after which they were washed to remove the PMA and rested for one day prior to their use.

6.1.1.2 *M. tuberculosis* auxotroph culture

For the purposes of these experiments an *Mtb* auxotroph strain, kindly provided by Associate Professor Digby Warner, was used. This attenuated, *Mtb* H37Rv-derived strain (mc^{2}6206), is unable to produce the essential nutrients leucine and pantothenate due to mutations inserted in the genes required for their production. Thus, this auxotrophic bacterium is alive but unable to replicate (547). Due to its severe attenuation, all manipulations with this bacterium can be performed in Biosafety Level 2 facilities.

Stock aliquots of mc^{2}6206 were stored at -80°C until required for experimental procedures. When required, one aliquot of bacteria (1ml, harvested at OD ≈ 0.8; 600nm) was thawed and added to 4ml Middlebrook 7H9 broth supplemented with OADC, 0.5% glycerol, 24mg

pantothenate and 50mg leucine (supplement concentrations used for 1000ml of broth). The initial culture was left undisturbed for 5 days at 37°C with 5% CO_2. Once bacteria had recovered from freeze/thaw shock, 1ml was taken and sub-cultured in 9ml fresh broth for a further 9 days, with the OD at 600nm measured every 2 days from day 5 to monitor growth. Once in the log growth phase (OD = 0.7-1.0), bacteria were either harvested and used for *in vitro* assays, or sub-cultured to ensure continued growth. To determine CFU counts, bacterial cultures or samples were plated on Middlebrook 7H9 broth supplemented as above, with bacterial agar powder added as the solidifying agent.

6.1.1.3 Fluorescent labelling of *Mtb*

Bacteria grown to log phase (as described above) were harvested and labelled with pHrodo™ Red, a pH-sensitive dye that fluoresces at low pH. Lyophilised pHrodo was reconstituted in DMSO per the manufacturer's instructions to generate a stock solution of 10.2mM. Bacteria were washed and re-suspended in FS 100mM sodium bicarbonate (pH = 8.5), and 2µl of the pHrodo stock solution added (final concentration of pHrodo = 51µM). The staining reaction was allowed to proceed at room temperature for 40 minutes in the dark, with gentle agitation. Bacteria were washed twice in FS 1X PBS (pH = 7.4) following this incubation step, and resuspended in FS 1X PBS (pH = 7.4) at the required concentration.

To confirm whether labelling of the bacteria had been successful, some bacteria were kept aside at the higher concentration used during staining. These bacteria were washed and resuspended in 0.1M citric acid/0.2M di-sodium hydrogen orthophosphate dehydrate buffers with pH values adjusted to 4.6, 6 and 7.6. Once resuspended, bacteria were analysed under a fluorescent microscope (ZEISS Axio Observer 7 with Colibri 7) for positive staining at low pH (4.6).

6.1.1.4 Opsonophagocytosis assay

Cells used for these assays were differentiated as described, and maintained in media supplemented with FBS. Prior to the start of the experiment, cells were washed twice and resuspended in media without FBS and without penicillin/streptomycin, to ensure no interference of these components with experimental outcomes. All subsequent steps requiring media were performed with serum-free media. Prior to the start of the assay, serum samples to be tested were heat inactivated at 56°C for 30 minutes to remove complement proteins; the positive control sample was not heat inactivated. Once bacteria were labelled (as described above) and sera heat inactivated, serum was added to bacteria

at 10% v/v (of final volume) and opsonisation allowed to occur for 30 minutes at room temperature, with gentle agitation. Once completed, the opsonised bacteria were diluted to the required concentration in FS 1X PBS. Bacteria were added to differentiated THP-1 cells (10:1 ratio of bacteria to cells), with triplicate wells per serum sample. Plates were incubated at 37°C with 5% CO_2 for 2 hours to allow time for phagocytosis. Cells were washed twice with pre-warmed media to remove extracellular bacteria; fresh media was added and the experiment allowed to run for a further 2 hours at 37°C with 5% CO_2. The differentiation procedure results in cell adherence, so a solution of 4mg/ml lidocaine 10mM EDTA in 1X PBS was used to lift cells (40-60 minutes at 37°C) prior to staining for FACS.

Once removed from the tissue culture plate, cells were stained for viability (LIVE/DEAD™ fixable Aqua staining kit; Life Technologies) and CD11b (Pacific blue, BD Pharmingen) expression to confirm differentiation. Cells were stained with the viability marker for 10 minutes at room temperature, followed by CD11b staining for 20 minutes at 4°C. Once stained, cells were fixed in 1% PFA for 20 minutes at 4°C. Staining and fixing steps were performed with samples protected from light. Once stained and fixed, samples were acquired on a BD™ LSR II flow cytometer, and data were analysed using the FlowJo® software package. The gating strategies used to identify pHrodo- and CD11b-labelled cells are presented below, as are individual experimental outcomes for the samples being investigated **(Fig. A)**.

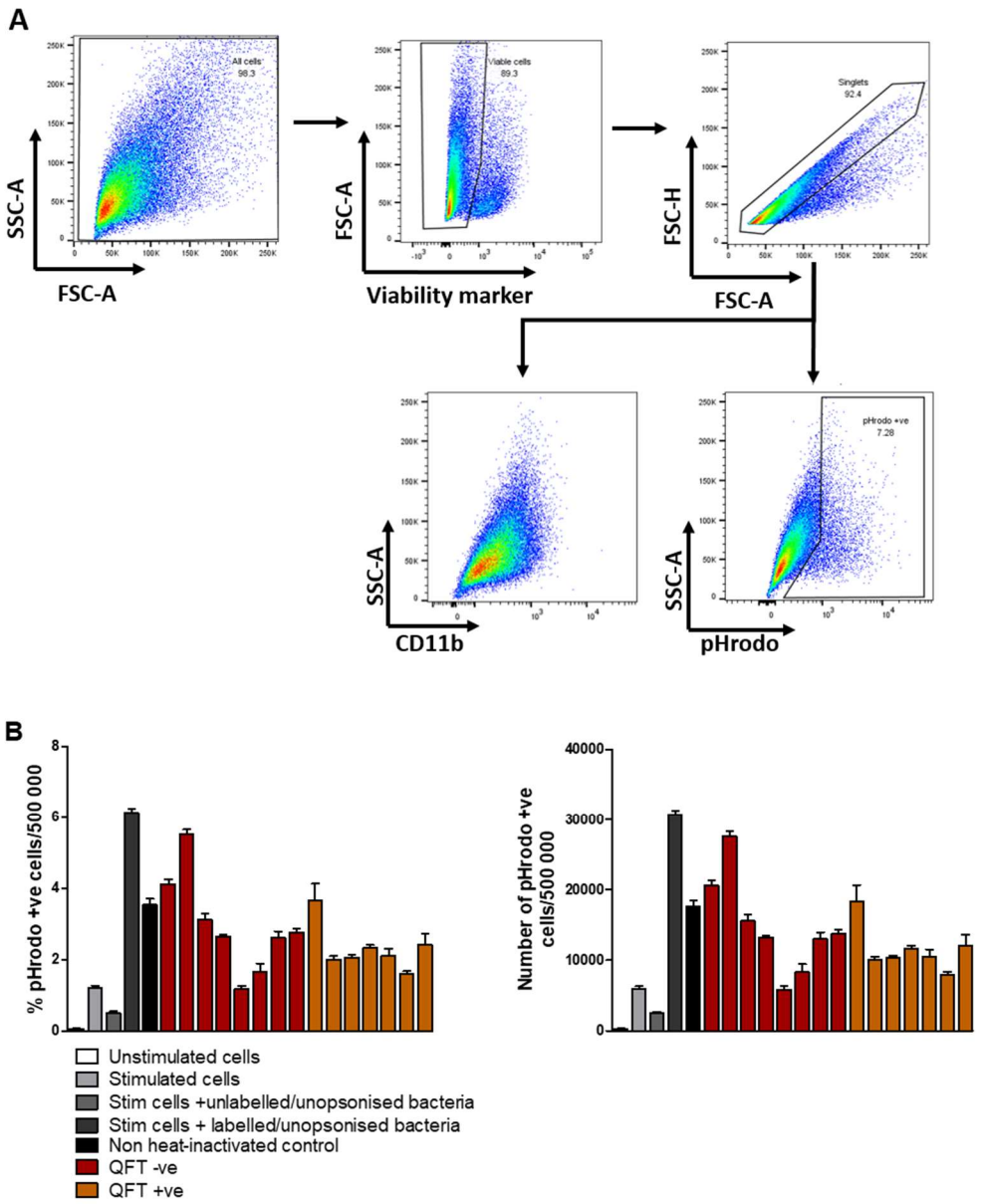

Figure A. Opsonophagocytosis assay gating strategy and individual sample outcomes. Forward scatter (cell size) and side scatter (cell granularity) parameters are used to identify differentiated THP-1 cells. Within this population, the LIVE/DEAD™ Aqua viability marker was used and within the live cell subpopulation the proportion of pHrodo™ positive cells and mean fluorescence intensity of CD11b were determined **(A)**. The proportion and number of pHrodo™ positive cells for individual experimental samples, as well as appropriate controls, were determined **(B)**. Data presented in **(B)** are an expansion of data presented in **Figure 5A**.

6.2 General Buffer Recipes

6.2.1 ELISA buffers

10X Phosphate-buffered saline (PBS)

80g NaCl

2g KCl

14.4g Na_2HPO_4 or 18g $Na_2HPO_4.2H_2O$

2.4g KH_2PO_4

Dissolve the above in 1L distilled H_2O, and adjust the pH to 7.2-7.4. To make 1X PBS, mix 100ml of 10X PBS with 900ml distilled H_2O. Solution can be filter sterilised; store at room temperature.

Carbonate coating buffer

1.6g Na_2CO_3

2.9g $NaHCO_3$

4.2g NaCl

Make up to 1L final volume using distilled H_2O, and adjust the pH to 9.5 using 1M citric acid. Solution can be filter sterilised if required; store at 4°C.

Blocking buffer

2g bovine serum albumin (BSA) OR 2g milk powder (2% w/v)

Dissolve in 100ml 1X PBS; store at 4°C.

Dilution buffer

1g BSA (1%w/v)

Dissolve in 100ml 1X PBS; store at 4°C.

Washing buffer (20X)

20g KCl

20g KH_2PO_4

144g $Na_2HPO_4.2H_2O$

800g NaCl

50ml Tween 20

Make solution up to 5L with distilled H_2O. To make 1X solution, mix 1L of the 20X solution with 19L distilled H_2O.

Substrate buffer (for use with p-Nitrophenyl phosphate (PNP) substrate powder)

0.2g NaN_3

0.8g $MgCl_2.6H_2O$

97 ml di-ethanolamine (liquefy in water bath if too viscous to use)

Add components to 700ml distilled H_2O, and adjust the pH to 9.8 with 10M HCl. Make up to 1L; store at 4°C.

PNP substrate

The substrate should be made up immediately prior to use. Measure volume of substrate buffer (described above) required for experiment. Weigh amount of PNP substrate powder required to yield a 1mg/ml solution; dissolve in substrate buffer and protect from light.

6.2.2 Mycobacterial culture solutions

Liquid growth medium

4.7g Middlebrook 7H9 broth powder (Difco™)

2ml glycerol

Add components to 900ml distilled H_2O. Autoclave solution for 10min (121°C and 100kPa). Let solution cool down to 50-55°C, and add 100ml (10%) OADC enrichment medium.

Agar plates

21g Middlebrook 7H11 agar powder (Difco™)

5ml glycerol

Add components to 900ml distilled H_2O. Autoclave solution for 10min (121°C and 100kPa). Let solution cool down to 50-55°C, and add 100ml (10%) OADC enrichment medium. To pour plates: work in a sterile tissue culture hood. Add 16ml agar to each petri dish while still warm. Leave lids off and allow to cool and set. Once cool, wrap stacks of plates in sterile plastic bags and store at 4°C until use (do not keep for more than one month and check for contamination prior to use).

6.2.3 Tissue processing and flow cytometry solutions

Complete DMEM (Dulbecco's modified Eagle medium - culture media)

500ml sterile DMEM (Gibco®, MA)

50ml sterile foetal bovine serum (FBS, heat inactivated) (Gibco®, MA)

2.5ml penicillin/streptomycin (200X) (Thermo Fisher Scientific, MA)

Add FBS and penicillin/streptomycin to DMEM and mix well. Filter sterilise (0.22µM filter) and store at 4°C; check for contamination prior to use.

Lung digestion buffer

0.002g DNase I (Roche, Germany)

0.02g Collagenase Type I (Gibco®, MA)

Add components to 150ml complete DMEM (as described above). Filter sterilise (0.22µM filter) and store at 4°C for up to 1 week.

Red cell lysis buffer (RCLB)

8.34g NH_4Cl

0.037g EDTA

1g $NaHCO_3$

Add components to 1L distilled H_2O. Filter sterilise (0.22µM filter) and store at 4°C.

MACS buffer

0.745g (2mM) EDTA

5g (0.5%) BSA

Add components to 1L 1X PBS. Filter sterilise (0.22µM filter) and store at 4°C.

Phorbol 12-myristate 12-acetate (PMA)

Freeze aliquots of purchased stock PMA at -20°C. When required for cell stimulation, prepare working solution in complete DMEM.

Ionomycin

Freeze aliquots of purchased stock Ionomycin at -20°C. When required for cell stimulation, prepare working solution in complete DMEM.

Permeabilization buffer

0.5g saponin

0.055g $CaCl_2$ or 0.073g $CaCl_2.2H_2O$

0.0625g $MgSO_4$

0.25g NaN_3

0.5g BSA

10mM HEPES (5ml of 1M solution)

Add components to 400ml 1X PBS and adjust pH to 7.4. Adjust volume to 500ml with 1X PBS. Filter sterilise (0.22µM filter) and store at 4°C.

Paraformaldehyde (PFA, 4%)

4g paraformaldehyde powder

Add powder to 100ml 1X PBS and allow to dissolve at 70°C. Once dissolved, allow to cool to room temperature and adjust the pH to 7.2. Store aliquots at -20°C until required.

6.2.4 Cell culture solutions

Complete RPMI-1640 medium

500ml sterile RPMI-1640 (Gibco®, MA)

50ml sterile FBS

2.5ml penicillin/streptomycin (200x)

Add FBS and penicillin/streptomycin to RPMI-1640 and mix well. Filter sterilise if desired (0.22µM filter) and store at 4°C; check for contamination prior to use.

PMA

Prepare PMA stock solution of 5mg/ml by dissolving powder in DMSO; freeze aliquots at -20°C. When required, prepare a 100nM PMA solution in RPMI-1640 medium for cell stimulation.

Cell lifting solution

Prepare a solution of 4mg/ml lidocaine-HCl, 10mM EDTA in sterile 1X PBS. Add this solution to wells to lift cells when desired.

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